Autoreceptor control of 5-HT release from central serotoninergic neurones

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AUTORECEPTOR CONTROL OF 5-HT RELEASE FROM CENTRAL SEROTONINERGIC NEURONES

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To my mother, for the love she has given me.

CONTENTS

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For all knowledge and wonder (which is the seed of knowledge) is an impression of pleasure in itself.

Francis Bacon

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It would be impossible to sum up in a few words my deep appreciation and respect for Dr. Peter Redfern who, throughout this study, was a model supervisor. In addition to his erudite, guidance and advice, Dr. Redfern will be a continuous source of inspiration throughout my career, and hopefully a continual friend.

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SUMMARY

The circadian variation in 5-hydroxytryptamine (5-HT) concentrations in brain tissue appear to result from circadian rhythms in both synthesis and release of the transmitter. The release of 5-HT is affected by many factors, including the activity of the terminal 5-HT autoreceptor which, in the rat brain, corresponds to the 5-HT_{1B} binding site. The results presented in this thesis show that this terminal 5-HT autoreceptor in the rat cerebral cortex and hippocampus does not display a circadian variation in its sensitivity. In the guinea-pig brain, the terminal 5-HT autoreceptor corresponds to the 5-HT_{1D} binding site. This receptor was also devoid of any circadian variation in its sensitivity.

The release of 5-HT can be modulated by the *alpha*$_2$-adrenoceptor located on 5-HT nerve terminals. The sensitivity of this receptor, when examined over 24-hours, did not reveal any significant circadian variation.

The somadendritic 5-HT_{1A} autoreceptor modulates the firing rate of 5-HT neurones and, consequently, the synthesis and the release of 5-HT in terminal-containing regions. The ability of this receptor to regulate 5-HT synthesis, when examined at mid-light and at mid-dark, did not display any differences in sensitivity.

Radio-ligand binding studies in the cerebral cortex did not reveal any significant circadian variation in either the total number of binding sites or the affinity of [^{3}H]5-HT binding to the 5-HT_1 binding site. Competition studies, using 8-OH-DPAT, suggest that the number of 5-HT_{1A} binding sites, corresponding to a postsynaptic receptor in this region, is unchanged during the light : dark cycle. Examination of [^{3}H]ketanserin binding to the 5-HT_2 binding site in the rat frontal cortex, at mid-light and mid-dark, did not reveal any significant differences.

It is concluded that the circadian variation in the release of 5-HT is not the result of a corresponding rhythm in the sensitivity of the terminal or the somadendritic 5-HT autoreceptor, but rather that they would be susceptible to, and capable of passing on, any circadian rhythm in the level of stimulus applied to them.

1 INTRODUCTION

1 INTRODUCTION

5-hydroxytryptamine (5-HT, serotonin) is a neurotransmitter in the central nervous system (CNS). Many aspects of its physiology vary over 24 hours. For example, a marked circadian variation in 5-HT concentrations is reported in several regions of the rat brain, values being higher during the light phase. In contrast, the synthesis and release of 5-HT are greater during the dark phase. Several studies have been carried out to identify the cause of this variation, which is discussed in the following sections, however, the sensitivity of the terminal- or the somadendritic-5-HT autoreceptor has not been investigated over 24 hours, these receptors are in a prominent position for regulating the activity of 5-HT neurones. In addition, receptors for other neurotransmitters have been identified on 5-HT nerve terminals, these receptors are capable of regulating the release of 5-HT. An example of such a receptor is the *alpha*$_2$-adrenoceptor, its sensitivity was also measured over 24 hours. In this way it is hoped to obtain some understanding of the contribution of these receptors, to the rhythm in the release and synthesis of 5-HT.

Radio-ligand binding studies were also performed during the light : dark cycle, to investigate for circadian changes in the affinity or the number of 5-HT$_1$ and 5-HT$_2$ binding sites.

It is the purpose of this introduction to give readers sufficient background in the published work in these areas, and thus allow them to evaluate the data presented in this thesis and the conclusions drawn from them.

1.1 CIRCADIAN RHYTHMS

The yearly rotation of the earth around the sun is responsible for the changes in seasons. Similarly, its rotation on its axis once every 24 hours determines the day-night cycle to which we are all exposed. Animals and plants have evolved over millions of years using these rhythms to regulate aspects of their physiology, biochemistry and behaviour, with periods ranging from milliseconds to several years.

Rhythms which approximate to a period of 24 hours are termed "circadian", in addition to these a variety of other rhythms exist. Rhythms with periods of less than 24 hours are termed ultradian (*e.g.* electrical activity in the brain), while those with periods much greater than 24 hours are termed infradian (*e.g.* the menstrual cycle), and those with periods of about one year are termed circannual (*e.g.* hibernation). It is not known whether all these rhythms are driven by the same oscillator as circadian rhythms.

This section is a brief introduction to circadian rhythms and their characteristics, and the reader is referred to comprehensive reviews elsewhere (Rusak and Zucker, 1979 ; Meijer and Reitveld, 1989).

1.1.1 Characteristics of circadian rhythms

Rhythms function to enable plants and animals to anticipate future events. A circadian rhythm (from the latin : circa = about ; diem = day) has been defined as "an oscillation in a biochemical, physiological or behavioural function which under conditions in nature has a period of exactly 24 hours, in phase with the environmental light and darkness, but which continues to oscillate under constant but permissive conditions of light and temperature, with a period of approximately but usually not exactly 24 hours" (Sweeney, 1975). The ability of circadian rhythms to persist under constant conditions, *i.e.* to free-run, suggests that they are endogenously generated. These rhythms are in no way fixed, but responsive to alterations of their time cues, resulting in phase shifts. Consequently, they will synchronise, or entrain, to an external time cue, so long as the period of the cue is close to 24 hours (Aschoff, 1978).

For animals, the natural environment provides a multitude of time cues which

function to synchronise rhythms. However, in the laboratory where the majority of time cues can be controlled, the light-dark cycle is considered to be the most important (Pittendrigh, 1981).

Ostensibly therefore, circadian rhythms are a feature of the adaptation of an organism to its environment. They function as homeostatic regulators of internal events and also extrinsic behaviours. They enable organisms to anticipate with some certainty recurring events and thus adapt appropriately.

1.1.2 Neural control of circadian rhythms

In mammals, the location of the endogenous rhythm generator remained largely hypothetical until 1967 when, using lesioning techniques, Richter (1967) discovered that localized lesions of the ventral hypothalamus caused the complete loss of free-running circadian rhythmicity. The precise area of the hypothalamus remained unknown for another 5 years, when further lesioning studies identified a structure in the anterior hypothalamus which was essential for circadian rhythmicity of adrenal corticosterone secretion (Moore and Eichler, 1972) and of drinking and locomotor activity (Stephan and Zucker, 1972).

The ability of the endogenous rhythm generator to be entrained by an external light-dark cycle suggested the existence of a neural connection between this generator and optic pathways of the retina. However, lesioning of the primary and accessory optic pathways (Chase *et al.* 1969 ; Moore and Eichler, 1972 ; Stephan and Zucker, 1972) failed to abolish entrainment, but it did lead investigators to the suprachiasmatic nuclei (SCN) of the hypothalamus. Subsequent autoradiographic studies in search of a neural connection revealed a direct bilateral neural pathway originating in the retina and terminating in the SCN (Hendrickson *et al.*, 1972). This pathway was named the retinohypothalamic tract (RHT). A second visual pathway is now known to link the retina to the SCN. This pathway is an indirect one, first supplying the ventral lateral geniculate nuclei (vLGN) and the intergeniculate leaflet (IGL), which subsequently send axons to the SCN (Swanson *et al.*, 1974 ; Pickard, 1985). This projection is called the geniculohypothalamic tract (GHT). Further experiments have shown the SCN to drive a great number of circadian rhythms in

rodents, *i.e.* sleep-wake cycle, locomotor activity, food intake, water intake, body temperature, adrenal corticosterone secretion and the rhythm in pineal melatonin (Rusak and Zucker, 1979 ; Moore and Eichler, 1972 ; Stephan and Zucker, 1972 ; Rosenwasser and Adler, 1986).

Transplantation studies of the SCN have reported the period of the circadian cycle of transplanted grafts to be maintained (Ralph *et al.*, 1990), thus conclusively proving that the SCN functions as a pacemaker and not just as a relay station or an oscillator. This finding is supported by the ability of surgically isolated SCN to retain electrical activity, while their removal abolished various circadian rhythms in rodents (Rusak and Zucker, 1979).

The SCN while functioning as the primary pacemaker is not the only rhythm generator in the central nervous system. Prolonged exposure to constant conditions or the bilateral lesioning of the SCN can induce a dissociation of free-running rhythms into two components. This phenomenon is known as "internal desynchronization" or "splitting". Examples of such phenomena include the bilateral lesioning of the SCN in the squirrel monkey which resulted in a loss of the circadian drinking rhythm but not the circadian body temperature rhythm (Fuller *et al.*, 1981). Similarly, humans in isolation, under constant conditions, have shown their free-running rhythms to split into two components, one group coupled to the sleep-wake cycle, and the other to the core body temperature (Aschoff, 1965 ; Wever, 1979 ; Czeisler *et al.*, 1980). Splitting is considered to expose two underlying circadian oscillators which have become out of phase with each other. The location of these secondary oscillators and their respective properties remain to be determined.

While secondary oscillators are observed during splitting, it is likely that they are not autonomous. Their innate rhythms are considered to be damped so that they may be driven by the primary pacemaker of the SCN (Turek, 1985).

1.1.3 Organization of the circadian pacemaker

The SCN receive two neural inputs from the retina, as already described, directly

via the RHT and indirectly *via* the vLGN and the IGL. It also receives inputs from the ventral subiculum and septal nuclei (Pickard, 1982), the periaqueductal grey, the paraventricular nucleus, the mid-brain, the retrochiasmatic area, the paraventricular thalamic nucleus and other hypothalamic areas (Moore, 1983) and a prominent projection from both the median and dorsal raphe nuclei (Dahlstrom and Fuxe, 1964 ; Azmitia and Segal, 1978).

The SCN projects efferent pathways to the majority of areas from which it receives inputs. However, two efferent pathways of the SCN terminate in areas from which arise, afferents to the SCN : one is the raphe (Bons *et al.*, 1983); and the other is the IGL (Watts *et al.* 1987). These feedback loops are considered to allow the SCN to adjust its own inputs.

1.1.4 Pharmacological control of circadian rhythms

In order to establish the nature of the circadian pacemaker in the SCN, several studies have been conducted to identify potential neurotransmitters associated with its function. While little is known about these putative neurotransmitters, several neurotransmitters have been identified and their manipulation has revealed valuable information about their potential role in the functioning of the SCN.

Transmitters located inside the SCN include vasopressin and neurophysin (Sofroniew and Weindl, 1978), somatostatin (Moore, 1983), gastrin-releasing peptide; bombesin (Roth *et al.*, 1982), vasoactive intestinal peptide (Card *et al.*, 1981), and corticotrophin-releasing factor (Palkovits *et al.*, 1983). The SCN also contain significant quantities of gamma-aminobutyric acid (GABA) and its synthetic enzyme glutamic acid decarboxylase (Perry *et al.*, 1971), suggesting that it is probably synthesized there. 5-hydroxytryptamine (5-HT) is also found in the SCN (Fuxe, 1965), together with its synthesizing enzyme, tryptophan hydroxylase (Brownstein *et al.*, 1975) and its metabolizing enzyme, monoamine oxidase (Saavedra *et al.*, 1974), as is the neurotransmitter acetylcholine (Murakami *et al.*, 1984), its synthesizing enzyme, choline acetyltransferase (Brownstein *et al.*, 1975) and nicotinic cholinergic receptors (Segal *et al.*, 1978).

Neurotransmitters located outside the SCN and which may act on the SCN include, 5-HT (Fuxe, 1965 ; Kiss *et al.*, 1984) which is thought to originate from efferents of the raphe and, neuropeptide Y, which is gaining recognition as the primary neurotransmitter of the GHT (Harrington *et al.*, 1985). In contrast, glutamate and aspartate are considered to be the neurotransmitters of the RHT, as they are released upon electrical stimulation of the optic nerve (Liou *et al.*, 1986).

Other neurotransmitters detected in axons of the SCN include dopamine, noradrenaline and adrenaline (Van den pol and Tsujimoto, 1985). However, they do not appear to be located selectively either inside or outside the SCN (Van den pol and Tsujimoto, 1985). The reader is referred elsewhere to a more comprehensive list of the neurotransmitters located inside and outside the SCN (Van den pol and Tsujimoto, 1985).

Selective transection of the GHT, or its associated nuclei, does not impair the ability of light to activate or suppress the SCN (Groos and Meijer, 1985). In contrast, selective transection or damage of the RHT is not possible, without risk of damage to the optic tract, which runs in close proximity.

Considerable evidence suggests that acetylcholine may be involved in relaying the information about the light-dark cycle to the SCN. Intraventricular administration of carbachol (a cholinergic receptor agonist) causes a small phase shift in blinded mice (Zatz and Herkenham, 1981), this effect is similar to that produced by light pulses. Also, light is reported to decrease the enzyme activity of N-acetyltransferase in the pineal gland; this effect is likely to be mediated by the RHT and the SCN (Rusak and Zucker, 1979), since carbachol injection close to the SCN resembles the effect of light, whilst an injection of *alpha*-bungarotoxin (nicotinic cholinergic receptor antagonist) prevents the effect of carbachol (Zatz and Brownstein, 1979). It is likely that carbachol is acting *via* nicotinic cholinergic receptors at the level of the SCN. In addition, the iontophoretic application of acetylcholine to the SCN produced excitation of 80% of SCN cells (Nishino and Koizumi, 1977). Although the above results suggest that acetylcholine may be the transmitter of the RHT, the SCN proved unresponsive to several other cholinergic agents but was inhibited by antagonists of excitatory amino acids (Cahill and Menaker, 1987).

Experiments monitoring the release of glutamate and aspartate from nerve terminals in the SCN showed their release to be effected by stimulation of the optic nerve (Liou *et al.*, 1986). In addition, the iontophoretic application of glutamate onto cells of the SCN, produces excitation (Nishino and Koizumi, 1977) and a phase shift, which resembles that of dark pulses (Meijer *et al.*, 1988). It is therefore probable that glutamate and aspartate serve as neurotransmitters of the RHT.

Studies employing benzodiazepines have proved useful in establishing the role played by the inhibitory neurotransmitter GABA; triazolam, a short acting benzodiazepine, produces phase shifts in locomotor activity. However, the similarity of its phase-response curves obtained during light and dark suggests that GABA may not be involved with the relaying of photic information to the SCN (Turek and Losee-Olson, 1986). Moreover, the phase shifting effect of triazolam is unaltered in animals that have been blinded (associated with a degenerated RHT) or pinealectomized (Van Reeth *et al.*, 1987). These findings suggest that the phase shifting effect of triazolam and probably GABA is not mediated by the RHT.

The depletion of brain serotonin using p-chlorophenylalanine (PCPA) has shown free running circadian rhythms in locomotor activity to disappear and then reappear after 7 days with an altered phase (Honma *et al.*, 1979). A tryptophan-free diet is associated with a marked reduction in brain 5-HT concentration, and a disturbance of the sleep-wake balance (Lanoir *et al.*, 1981). However, circadian rhythms in locomotor activity (Block and Zucker, 1976) and prolactin levels (Dunn *et al.*, 1980) are capable of persisting following raphe lesioning. In addition, transection of the raphe afferents of the SCN, which would be expected to have similar effects to lesioning of the raphe nuclei, was found not to alter entrained or free-running rhythms (Inouye and Kawamura, 1980). In contrast, the iontophoretic application of 5-HT or the electrical stimulation of the raphe nuclei inhibits the firing of SCN cells (Groos *et al.*, 1983). These findings suggest that the raphe nuclei are not primarily involved in the generation or entrainment to a particular light : dark cycle but that they are able to modulate the expression of circadian rhythms. In addition, the raphe nuclei receive retinal afferents (Foote *et al.*, 1978), it is thought that these afferents are not in themselves involved in the acclimatization to a particular light dark

cycle but contribute to the generation and modulation of a coherent circadian rhythm.

In vivo voltammetry has recently demonstrated the existence of a circadian variation in extracellular concentrations of 5-hydroxyindoleacetic acid (5-HIAA) (Faradji *et al.*, 1983 ; Martin and Marsden, 1985), in the SCN of the rat. 5-HIAA is the major metabolite of 5-HT and is reported to be a good indicator of changes in 5-HT released (Sharp *et al.*, 1984). These findings imply that extracellular 5-HT concentrations are higher during the dark period, when the animals are most active, suggesting that the release of 5-HT into the synaptic cleft is greater at this time (Martin and Marsden, 1985 ; Kalen *et al.*, 1989). Electrophysiological studies have shown the firing rate of 5-HT neurones to be higher during the dark phase, when the animals are active, and this may account for the greater 5-HT release at this time (McGinty and Harper, 1976 ; Trulson and Jacobs, 1979). In contrast, the firing rate of the SCN is at its lowest during the dark phase (Inouye and Kawamura, 1980). Meijer and Groos (1988) demonstrated microiontophoretically applied 5-HT to inhibit the neuronal firing of about one-half of the SCN and vLGN cells. It is tempting to speculate that the circadian variation in raphe neuronal firing and 5-HT release at the SCN, functions to modulate the neuronal firing activity of the SCN, but it is more probable that this input from the raphe nuclei is one of many which serves to enable the SCN to control and co-ordinate a variety of circadian rhythms.

1.2 CIRCADIAN RHYTHMS IN PARAMETERS OF CNS 5-HT FUNCTION

Research into this aspect of 5-HT has generated a vast amount of data, and it is beyond the scope of this section to cover it in detail. The following sections give brief summaries of some of the results that have been obtained.

1.2.1 Anatomical distribution of 5-HT in the central nervous system

Significant quantities (0.1-0.36 μg/g of tissue) of the monoamine 5-HT were first reported in mammalian brain by Twarog and Page (1953). It was found primarily in the mesencephalon (midbrain) and the diencephalon (hypothalamus and thalamus); the highest concentrations were found in the hypothalamus and the caudate nucleus, and the lowest in the cerebellum (Amin *et al.*, 1954 ; Bertler and Rosengren, 1959).

The advent of fluorescence histochemistry enabled the mapping of 5-HT containing neurones in the brain (Falck *et al.*, 1962). More recently, the development of other methods (*e.g.* autoradiography and immunohistochemistry) has contributed to a more detailed visualization of the fine structure and organization of 5-HT neurones in the CNS (Steinbusch and Nieuwenhuys, 1981).

The nine nuclei containing 5-HT cell bodies from which 5-HT neurones originate, are located in the medulla oblongata, pons and mesencephalon. These cell bodies are commonly referred to as raphe nuclei. They give rise to both ascending and descending neuronal pathways. The more caudal groups of nuclei project descending pathways to the medulla and spinal cord, whereas the more rostral 5-HT cell groups (dorsal, median and central raphe) project ascending pathways to innervate the amygdala, hypothalamus and thalamus, the hippocampus, olfactory bulb and tubercle, the SCN and the cerebral cortex.

1.2.2 Biosynthesis and catabolism of 5-HT

5-hydroxytryptamine is unable to cross the blood brain barrier and is synthesized in the brain from the amino acid L-tryptophan (obtained primarily from the diet).

L-tryptophan is transported from the plasma into neurones by an active uptake process. This process is also responsible for the uptake of a number of other neutral

amino acids (*e.g.* tyrosine, phenylalanine, leucine, isoleucine, valine, methionine and histidine) and, therefore, L-tryptophan uptake is a competitive process.

Once inside the neurone, L-tryptophan is hydroxylated at the 5-position of the indole ring to form 5-hydroxytryptophan (5-HTP). The hydroxylation is catalysed by the enzyme tryptophan hydroxylase, which requires molecular oxygen and a pterin cofactor as an electron donor. Once synthesized, 5-HTP is decarboxylated almost immediately to 5-HT, by the enzyme 5-hydroxytryptophan decarboxylase, and then stored in a bound form inside synaptic vesicles.

The enzyme responsible for the catabolism of 5-HT is monoamine oxidase (MAO), which oxidizes the amino group to form the aldehyde, 5-hydroxyindole acetaldehyde, and this can be oxidized further to 5-hydroxyindoleacetic acid (5-HIAA).

In the pineal gland, 5-HT is converted readily to melatonin, the reaction being catalysed by two enzymes. The first, N-acetyltransferase, acetylates 5-HT to form N-acetylserotonin, and the second, 5-hydroxyindole-o-methyl transferase, which requires S-adenosyl methionine as the methyl donor, methylates N-acetylserotonin to form melatonin.

The concentrations of both 5-HT and melatonin in the pineal gland display a circadian variation. These rhythms are considered to be under the control of the SCN (see Neural control of circadian rhythms). The SCN are thought to act *via* the sympathetic nervous system to regulate the activity of N-acetyltransferase.

1.2.3 Circadian variation in 5-HT concentrations

A circadian variation in the concentration of 5-HT was first reported for the mouse brain (Albrecht *et al.*, 1956). Since then, a similar rhythm has been found to exist in the brains of a variety of species including rat (Quay, 1965), cat (Reis *et al.*, 1969) and ferret (Yates and Herbert, 1979). The first studies measured whole brain 5-HT concentrations, whereas later studies have focused on measuring 5-HT concentrations in a number of brain regions including, frontal cortex, hypothalamus, dorsal and median raphe nuclei and the pineal gland (Quay, 1965,1968 ; Okada, 1971 ; Hery *et al.*, 1972 ; Hillier and Redfern 1976b ; Semba *et al.*, 1984 ; Agren *et al.*, 1986). Moreover, the rhythm in

5-HT concentrations of the dorsal raphe persists under constant conditions suggesting that it is endogenously generated (Semba *et al.*, 1984). In contrast, a recent study has demonstrated that the rhythm in hypothalamic 5-HT concentrations, in hamster brain is not circadian in nature and may be driven by the light : dark cycle (Ferraro and Steger, 1990).

The amplitude of the circadian variation in 5-HT concentrations varies from region to region (in the brain), and is at its highest in the frontal cortex, hypothalamus and the lower brainstem (Quay, 1968). A rhythm is also observed in the SCN (Martin and Marsden, 1985), where the concentrations of 5-HT during the light phase are double those seen during the dark phase. Although these studies demonstrate that 5-HT concentrations peak during the light phase, they are nevertheless, asynchronous (*i.e.* the phase of the rhythms is not identical in all brain regions).

In order to understand the reasons for the marked changes in 5-HT concentrations observed during the light-dark cycle, much effort has been focused on the components of the 5-HT synthesis pathway, since it is thought that a circadian variation in one or more of these components may reflect the circadian variation of 5-HT concentrations. The following section discusses some of the results obtained from the analysis of the components of the 5-HT synthesis pathway.

1.2.4 Tryptophan availability

The amino acid L-tryptophan is the precursor of 5-HT synthesis. It is converted by the enzyme tryptophan hydroxylase to 5-hydroxytryptophan. Experiments of Fernstrom and Wurtman (1971) demonstrated that small changes in plasma and brain tryptophan, which occur during physiological conditions, could lead to a rapid elevation of brain 5-HT concentrations. Other authors have also reported a circadian variation in the availability of tryptophan in both plasma and brain (Rapoport *et al.*, 1966 ; Martin and Redfern, 1982). Thus the availability of tryptophan may contribute in part to the changes in 5-HT concentrations seen over 24 hours. However, under normal physiological conditions, the concentrations of plasma and brain tryptophan are highest during the dark phase (Fernstrom and Wurtman, 1971), and are thus 180° out of phase with the rhythm of

5-HT concentrations, which are highest during the light phase. Further evidence against tryptophan as the prime determinant is presented from the measurement of tryptophan concentrations in the cerebrospinal fluid (CSF) (by *in vivo* microdialysis); no significant change in tryptophan concentrations was seen over 24 hours (Hutson *et al.*, 1984). It is likely, therefore, that tryptophan availability is not directly responsible for the circadian variation seen in 5-HT concentrations, although it can have an effect.

1.2.5 Tryptophan hydroxylase activity

This enzyme is the rate-limiting step in the intraneuronal conversion of tryptophan to 5-HT (Ashcroft *et al.*, 1965), consequently variation in its activity may account for the circadian variation in 5-HT concentrations. However, the results obtained by a number of groups are inconclusive. For example, a significant circadian variation was first reported by Kan *et al.* (1977) in the rat, and by Natali *et al.* (1980) in the mouse. The presence of such a rhythm was confirmed for the rat by Cahill and Ehret (1981) and Redfern and Sinei (1985). The enzyme activity is highest during the dark phase, opposite to the peak in 5-HT concentrations.

Other groups, however, were unable to detect a variation in the activity of the enzyme, using similar techniques (Deguchi, 1977 ; McLennan and Lees, 1978). Two equally plausible explanations have been put forward to account for this discrepancy. Firstly, a circadian variation in enzyme activity has been reported by workers who have used specific brain regions, as opposed to whole brain preparations where no rhythms could be detected. Therefore, since the phase of the enzyme activity rhythm has been shown to vary from region to region (Natali *et al.*, 1980), it is likely that the use of whole brain preparations would decrease the possibility of detecting such a rhythm. Secondly, the process of homogenization is considered to alter tryptophan hydroxylase activity, since enzyme activity in brain homogenates was considerably higher than that found *in vivo* (Kizer *et al.*, 1976 ; Lin *et al.*, 1969). It has been proposed that homogenization, by mimicking physiological depolarization, maximally activates tryptophan hydroxylase (Elks *et al.*, 1979). In addition, the terminal 5-HT autoreceptor may be involved in the regulation of tryptophan hydroxylase activity (Hjorth and Magnusson, 1988).

1.2.6 5-hydroxytryptophan decarboxylase activity

Once synthesized, 5-HTP is decarboxylated almost immediately by the enzyme 5-hydroxytryptophan decarboxylase (5-HTP-decarboxylase) to 5-HT. This enzyme is highly active and is located inside monoamine neurones; it is also involved in the synthesis of both dopamine and noradrenaline (Anden *et al.*, 1965). There is evidence to suggest that different enzymes are involved in the decarboxylation of 5-HTP and L-dihydroxyphenylalanine (DOPA). The intracisternal administration of 6-hydroxydopamine preferentially reduces DOPA-decarboxylase activity but not 5-HTP-decarboxylase activity (Bloom *et al.*, 1969 ; Breese and Traylor, 1970). In addition, Sims *et al.* (1973) found differences in kinetic properties, ease of denaturation, reliance of activity on the availability of co-factors, and the relative distribution in the brain of the two enzymes. It is therefore likely that the enzymes responsible for the decarboxylation of 5-HTP and DOPA are functionally different, yet they both possess the capacity of non-selectively decarboxylating 5-HTP or DOPA. Selectivity is probably achieved by the specific localization of 5-HTP-decarboxylase to 5-HT neurones and DOPA-decarboxylase to dopaminergic and noradrenergic neurones.

5-HTP-decarboxylase activity in crude brain homogenates varies over 24 hours, while that of the purified enzyme remains unchanged (Hillier and Redfern, 1976a). This variation in the activity of the enzyme, obtained from brain homogenates, was attributed to other factors, such as substrate competition or co-factor availability. The authors also showed that this enzyme is normally unsaturated, thus suggesting that it probably contributes little to the circadian variation in 5-HT concentrations.

1.2.7 5-HT release

Hery *et al.* (1972) first demonstrated that 5-HT release is greater during the dark phase, they also found that newly synthesized 5-HT was released in preference to the old. Their results, however, were rather difficult to interpret because they had not differentiated between 5-HIAA derived from the intraneuronal metabolism of 5-HT or that from neuronally released 5-HT.

Recent studies have used *in vivo* voltammetry to measure 5-HIAA concentrations over 24 hours, in the caudate nucleus, cerebral cortex, hippocampus, raphe nuclei (Cespuglio *et al.*, 1983) and the SCN (Faradji *et al.*, 1983 ; Martin and Marsden, 1985) of the rat, as 5-HIAA concentrations are a good indicator of 5-HT released (Faradji *et al.*, 1983 ; Sharp *et al.*, 1984). Martin and Marsden (1985) correlated their results of *in vivo* voltammetry with intracerebral microdialysis studies measuring extracellular 5-HT concentrations (release into the synaptic cleft) in the hypothalamus, while Faradji *et al.* (1983) correlated 5-HIAA concentrations with locomotor activity. The results from these studies suggest that extracellular 5-HIAA concentrations are higher during the dark phase, and also that the greatest change occurs during transition from light to dark (increase) and from dark to light (decrease). In support, microdialysis studies have confirmed that 5-HT release rates are higher during the dark phase in the hypothalamus (Martin and Marsden, 1985) and the hippocampus (Kalen *et al.*, 1989). Furthermore, the inability of Kalen *et al.* (1989) to observe corresponding changes in 5-HIAA concentrations may be a reflection of the use of a 5-HT re-uptake inhibitor in the perfusion fluid.

In general, these findings suggest that the functional activity of serotoninergic neurones is highest during the dark phase, *i.e.* when 5-HT concentrations are at their lowest, implying that 5-HT concentrations do not function to regulate the release of 5-HT into the synaptic cleft.

1.2.8 5-HT uptake

The action of released 5-HT is terminated by its re-uptake into presynaptic nerve endings, where it is either degraded intraneuronally by MAO or reincorporated into synaptic vesicles. Meyer and Quay (1976) found that the *in vitro* uptake of 5-HT, by slices of the SCN, displays a circadian variation, with highest uptake taking place during the dark phase, and lowest during the light phase, while in the hypothalamus, they observed a rather complex rhythm which was characterized by three periods of high uptake mingled with three periods of low uptake. However, recent studies by Rovescalli *et al.* (1989) investigating the imipramine binding and the uptake of 5-HT by slices of the hypothalamus and cerebral cortex, during light and dark, reported a difference in both parameters only

for the hypothalamus, with imipramine binding and 5-HT uptake being greater during the dark. Wirz-Justice *et al.* (1983) also found imipramine binding in the SCN to display a similar rhythm. The imipramine, or antidepressant binding site, and the 5-HT uptake site, resemble each other and it was recently proposed that the 5-HT uptake inhibitor, paroxetine, binds to a single non-heterogeneous site which resembles the antidepressant binding site (Marccusson *et al.*, 1989). On this basis these authors proposed that the imipramine binding site and the 5-HT uptake site are part of the same complex, or even one and the same.

The rhythm in 5-HT uptake is in phase with that of 5-HT release, as would be expected, since re-uptake is the primary means of terminating transmitter action, which would need to be at its most active when 5-HT neurones themselves are most active.

1.2.9 Functional significance of the circadian variation in 5-HT concentrations

Many of the factors involved in the biosynthesis process of 5-HT display a circadian variation, but their phases appear to be inconsistent with being directly responsible for the circadian variation seen in 5-HT concentrations. A few of these biosynthetic steps possess suitable characteristics which would enable them to act as regulators of neurotransmitter function. Dietary influences are shown to be important in determining CNS concentrations of tryptophan, whereas the rhythm in activity of the enzyme tryptophan hydroxylase in being 180° out of phase with 5-HT concentrations is considered unlikely to be able to adapt quickly to meet functional requirements. The enzyme 5-HTP-decarboxylase is susceptible to a variety of influences that may be part of a control mechanism for 5-HT function but the fact that this enzyme is normally unsaturated with substrate suggests that it is not of controlling importance. It may well be that 5-HT functions in the CNS as a general modulator, and that precise control of its activity is not desirable. All the factors mentioned are likely to contribute to the overall activity of 5-HT. On the other hand, it seems unlikely that changes in the functional activity of 5-HT neurones are a direct result of changes in 5-HT concentration. It is more

plausible that the observed changes in presynaptic function are designed to replenish stores of transmitter that are used at various rates over 24 hours.

It is likely, therefore, that the changes in 5-HT release are partly responsible for the observed daily fluctuations in 5-HT concentrations. However, the situation is further complicated by the fact that 5-HT reuptake is also highest when release is highest. This seems to suggest that 5-HT release has little effect on 5-HT concentrations.

It has been demonstrated recently that there exist 5-HT receptors on presynaptic 5-HT nerve terminals. These receptors are activated by the 5-HT released from their corresponding nerve terminals, and are therefore termed 5-HT autoreceptors. They function by feedback inhibition to regulate release and possibly synthesis of 5-HT. In this way they could be important regulators in controlling the circadian oscillation in presynaptic activity. It is the primary aim of this thesis to assess how 5-HT autoreceptor activity changes during the light : dark cycle.

1.3 RECEPTORS FOR 5-HT

Gaddum and Picarelli (1957) demonstrated the existence of two distinct 5-HT receptors, which they termed "D" and "M", based on the selective activity of the antagonists, dibenyline and morphine. Since this early classification, the identification of 5-HT receptor subtypes has proliferated, largely helped by the development of more selective compounds and the increased sensitivity of biochemical techniques. The true classification and organization is still rather confusing, and made more so by the lack of a concerted standardized nomenclature.

1.3.1 Electrophysiological evidence for multiple 5-HT receptors

The technique of microiontophoresis has allowed the testing of agents, for example 5-HT, that do not permeate the blood-brain barrier. From the results of this technique, Haigler and Aghajanian (1977) proposed the existence of three distinct 5-HT receptors based on the neuronal responses to 5-HT; Aghajanian (1981) designated these receptors S_1, S_2 and S_3.

S_1

This receptor was identified from unit recordings of facial motor neurones (McCall and Aghajanian, 1979) and spinal motor neurones (White and Neuman, 1980). The activation of this postsynaptic receptor by 5-HT, and 5-HT agonists, facilitates the depolarizing action of the excitatory amino acid glutamate. The facilitatory actions of 5-HT are blocked by small doses of classical 5-HT antagonists, such as cyproheptadine, methysergide and metergoline (McCall and Aghajanian, 1979,1980).

S_2

This receptor is located on either the soma or dendrites of raphe neurones. Its activation results in reduction of the firing activity of 5-HT neurones. Whilst being extremely sensitive to the action of LSD, this receptor is completely insensitive to the classical 5-HT antagonists (Aghajanian, 1981). It is considered to be the somadendritic autoreceptor (Aghajanian, 1981), due to its location and its ability to regulate the activity of

serotoninergic neurones.

S_3

This receptor is located postsynaptically in the forebrain areas receiving a dense 5-HT input, for example, ventral lateral geniculate and the amygdala. Microiontophoretically applied 5-HT produces a depression in the firing rate. LSD is a weak agonist at this site, whereas the classical antagonists have little effect (Haigler and Aghajanian, 1974 ; Aghajanian, 1981).

1.3.2 Radio-ligand binding evidence for multiple 5-HT binding sites

Peroutka and Snyder (1979) were the first authors to propose the existence of two major populations of central 5-HT binding sites. The sites labelled by nanomolar concentrations of [^{3}H]5-HT were designated "5-HT_1", whilst those labelled by nanomolar concentrations of [^{3}H]spiperone (a neuroleptic used in dopamine binding studies) were designated "5-HT_2". In contrast, lysergic acid diethylamide (LSD) displayed an equal affinity for both sites. These binding sites were observed to display a marked regional variation in binding densities, adding further support to the idea that they represent distinct molecular entities (Peroutka and Snyder, 1981).

Recently, a further 5-HT binding site has been identified in the CNS. This site was termed 5-HT_3; it displays an intermediate affinity for 5-HT, in between that found for the 5-HT_1 and the 5-HT_2 sites. Its discovery is largely attributed to the development of potent and selective agents (MDL 72222, GR 38032F "ondansetron" and ICS 205 930) whose properties differ from those of existing compounds for 5-HT_1 and 5-HT_2 sites (Kilpatrick *et al.*, 1987 ; Watling, 1988).

1.3.2.1 5-HT_1

In 1981, Pedigo *et al.* provided evidence to suggest that the 5-HT_1 binding site was heterogeneous, based on the ability of spiperone to displace [^{3}H]5-HT binding in a biphasic manner. Binding sites displaying a high and a low affinity for spiperone were termed 5-HT_{1A} and 5-HT_{1B} respectively. Further support for this heterogeneity in 5-HT_1 binding sites was provided by Schnellmann *et al.* (1984).

A more comprehensive distinction between these sites came with the use of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (Arvidsson *et al.*, 1981), a compound shown to be a 1000 fold more selective for the 5-HT_{1A} binding site (Middlemiss and Fozard, 1983). Several binding studies have been performed using [^{3}H]8-OH-DPAT (Gozlan *et al.*, 1983 ; Hoyer *et al.*, 1985 ; Palacios *et al.*, 1987). Autoradiographic studies revealed a heterogeneous distribution of the 5-HT_{1A} binding sites in the brain (Marcinkiewicz *et al.*, 1984 ; Pazos and Palacios, 1985), with the highest density of these binding sites being found in the hippocampus, septum, cortex, raphe and the striatum (Gozlan *et al.*, 1983 ; Cortes *et al.*, 1984 ; Hall *et al.*, 1985 ; Verge *et al.*, 1986).

The selective destruction of serotoninergic nerve terminals by the intracerebral administration of the neurotoxin 5,7-DHT was associated with an unaltered number of 5-HT_{1A} binding sites in any region (Verge *et al.*, 1986 ; Crino *et al.*, 1990), whereas the destruction of intrinsic hippocampal neurones by the local injection of kianic acid, produced a marked reduction of the number of the hippocampal 5-HT_{1A} sites (Hall *et al.*, 1985), and the destruction of cortical neurones by ibotenic acid, reduced the number of cortical 5-HT_{1A} sites (Crino *et al.*, 1990), suggesting that these 5-HT_{1A} binding sites are located postsynaptically.

When 5,7-DHT was used to lesion serotoninergic cell bodies of the dorsal raphe, their degeneration was associated with a marked reduction in [^{3}H]5-HT and [^{3}H]8-OH-DPAT binding sites, suggesting the presynaptic location of these binding sites on serotoninergic cell bodies and/or dendrites (Verge *et al.*, 1986 ; Thor *et al.*, 1990). In addition, a similar treatment was associated with a reduction in the number of [^{3}H]8-OH-DPAT binding sites in the striatum (Gozlan *et al.*, 1983 ; Hall *et al.*, 1985). It was suggested therefore that this presynaptic site may be similar to that found on raphe cell bodies. However, this does not seem to be the case since the pharmacological properties of the raphe 5-HT_{1A} sites are different. In particular, the raphe 5-HT_{1A} sites exhibited a higher affinity for [^{3}H]8-OH-DPAT, and this binding was sensitive to the presence of GTP analogues (Hall *et al.*, 1985 ; Verge *et al.*, 1986). Furthermore, [^{3}H]8-OH-DPAT does not bind to the terminal 5-HT autoreceptor in the striatum (Middlemiss, 1985b). Moreover, under the assay conditions of Gozlan *et al.* (1983) it was considered to have bound to the 5-HT re-uptake site since binding was prone to displacement by a number of

selective 5-HT uptake inhibitors (Schoemaker and Langer, 1986).

Indeed, [^{3}H]8-OH-DPAT is considered to bind to a single binding site in the membranes of rat, pig and human cortex and hippocampus. The affinity values and the rank order of potency of a variety of compounds in displacing [^{3}H]8-OH-DPAT binding from this site were indistinguishable in these species. Also, the correlation between these species suggests that [^{3}H]8-OH-DPAT binds to the same site in all three species. Moreover, its anatomical distribution in human brain resembles that found in rat and mouse brain (Palacios *et al.*, 1987).

High numbers of the 5-HT$_{1B}$ binding sites are found in the caudate nucleus, globus pallidus, subiculum and the substantia nigra (Cortes *et al.*, 1984 ; Pazos and Palacios, 1985 ; Verge *et al.*, 1986). These 5-HT$_{1B}$ binding sites are located both, pre- and post-synaptically on serotoninergic neurones, on the grounds that, transection of afferent axons or DRN ablation, or the local injection of ibotenic acid is associated with a reduction in their number (Crino *et al.*, 1990). In addition, the local injection of 5,7-DHT is reported to produce, both a reduction in the number of non-5-HT$_{1A}$ sites (presumably 5-HT$_{1B}$), in the substantia nigra (Verge *et al.*, 1986), and an increase in the number of 5-HT$_{1B}$ binding sites in the caudate putamen (up-regulation of post-synaptic 5-HT$_{1B}$) (Offord *et al.*, 1988). The 5-HT$_{1B}$ binding site has been identified only in rat and mouse brain, and is absent in guinea-pig, human, pig, pigeon and a number of other species (Heuring *et al.*, 1986 ; Hoyer *et al.*, 1986a ; Martial *et al.*, 1989 ; Waeber *et al.*, 1989).

Autoradiographic studies of Cortes *et al.* (1984) first identified a binding site in rat choroid plexus that could be labelled by [^{3}H]5-HT, [^{3}H]LSD or [^{3}H]mesulergine. Its characteristics were distinct with respect to the rank order of potencies of agents with affinities for the 5-HT$_{1A}$, 5-HT$_{1B}$ or the 5-HT$_2$ sites, and therefore it was designated the "5-HT$_{1C}$ site". A similar pharmacological binding site has been identified in pig cortex and choroid plexus (Pazos *et al.*, 1984), human cortex, hippocampus and choroid plexus (Hoyer *et al.*, 1986b), thus implying that this site may well be conserved within these species. This site displays certain characteristics which resemble the 5-HT$_2$ binding site, suggesting that the two may be related. The reader is referred to a recent review on this controversy (Hoyer, 1988).

A further 5-HT binding site was identified in bovine brain membranes, using [^{3}H]5-

HT in the presence of 100 nM 8-OH-DPAT and 100 nM mesulergine, a condition designed to inhibit over 90% of specific binding to the 5-HT_{1A} and the 5-HT_{1C} site (Heuring and Peroutka, 1987). A considerable amount of [^{3}H]5-HT binding was still evident under these conditions. This binding site was distinct pharmacologically from those already identified and was designated the "5-HT_{1D}" binding site. Herrick-Davis and Titeler, (1988) and Waeber *et al.* (1988), using similar blocking conditions demonstrated this site to be present in pig, rat, calf and human brain. Although all five species possess this site, they do so in different proportions, the highest being found in bovine and human brain, where it is the most common 5-HT_1 binding site, whereas it represents only a small proportion in rat brain.

In the presence of pharmacologically blocked 5-HT_{1A} and 5-HT_{1C} sites (remembering that there are no 5-HT_{1B} sites in bovine brain), 5-carboxyamidotryptamine (5-CT) produced a biphasic displacement of [^{3}H]5-HT binding, suggestive of the heterogeneity of the 5-HT_{1D} binding site (Titeler and Herrick-Davis, 1988). Under similar conditions, 5-CT, ergotamine or GR 43175 can selectively displace [^{3}H]5-HT binding from the high-affinity component, leaving the [^{3}H]5-HT still bound to the low-affinity site (Leonhardt *et al.*, 1989 ; Sumner and Humphrey, 1989). This low-affinity [^{3}H]5-HT binding site was designated "5-HT_{1E}". It is also identified in human, pig and rat brain membranes, however, the numbers found in rat are much lower than those found in human brain (Titeler and Herrick-Davis, 1988 ; Leonhardt *et al.*, 1989 ; Sumner and Humphrey, 1989).

1.3.2.2. 5-HT_2

The development of ketanserin has enabled the 5-HT_2 binding sites to be correlated with the "D" receptor, first described by Gaddum and Picarelli (1957). Spiperone was one of the first ligands to be used to bind these sites (Peroutka and Snyder, 1979), however, its lack of selectivity soon led to the development of [^{3}H]ketanserin, an antagonist exhibiting a high affinity and selectivity for the 5-HT_2 sites (Leysen *et al.*, 1981,1982). Using this radioligand, the 5-HT_2 binding sites were demonstrated to be concentrated in the frontal cortex of the rat and guinea-pig brain (Leysen *et al.*, 1982). A similar 5-HT_2 binding site was also identified in human and pig brain cortex (Hoyer *et al.*, 1986b). In

addition to binding the 5-HT_2 sites, [^{3}H]ketanserin also exhibits an affinity for *alpha*$_1$-adrenergic and H_1-*histaminergic* sites; its binding to these sites is considered to be minimal when used at low concentrations (Leysen *et al.*, 1981). Furthermore, [^{3}H]ketanserin binding to the 5-HT_2 sites was displaced by a number of serotonin agonists and antagonists; antagonists generally revealed IC_{50} values in the nanomolar range, whereas agonists exhibit IC_{50} values of micromolar order (Leysen *et al.*, 1982), adding further support to the initial subdivision of 5-HT binding sites as proposed by Peroutka and Snyder (1979).

Recently it was suggested that the rat cortex contains both a high and a low affinity state of the 5-HT_2 binding site (Titeler *et al.*, 1985,1987), with the agonist radioligand [^{3}H]4-bromo-2,5-dimethoxyamphetamine ([^{3}H]DOB) labelling the high affinity state, and the antagonist radioligand [^{3}H]ketanserin labelling both the high and the low affinity states. However, Pierce and Peroutka (1989) suggested that the 5-HT_2 agonist [^{3}H]DOB labels a distinct 5-HT_2 binding "subtype" as opposed to a "state". Their evidence was derived from the six fold difference in affinity of 5-HT to displace [^{3}H]ketanserin binding from rat and human cortex as opposed to bovine cortex, the Hill coefficient for this displacement being significantly less than unity for rat and human cortex, while that for bovine cortex was essentially unity. On the basis of these results, Pierce and Peroutka (1989) proposed that the high affinity site labelled by [^{3}H]DOB (found in rat and human cortex) be designated "5-HT_{2A}", while the low affinity site, labelled by [^{3}H]ketanserin and found in all three species, be designated "5-HT_{2B}". In contrast, Leonhardt and Titeler (1989) observed little difference in the Hill values from these three species, all being considerably less than unity.

The inability of both groups to demonstrate specific [^{3}H]DOB binding in bovine cortex is considered to be due, partly, to the low density of the high affinity 5-HT_2 binding sites in this species, to the ability of [^{3}H]DOB to label only a small fraction of these sites, and to the low specific radioactivity of this ligand. In addition, Strange (1990) proposed that the apparent absence of [^{3}H]DOB binding could result from a weakened interaction with a G protein. Furthermore, there was a significant difference in the IC_{50} value of 5-HT in displacing [^{3}H]ketanserin binding from human and bovine cortex. However, the most important difference between the results from the two laboratories was considered to be

the positioning of the "datum" point for the displacing effect of 5-HT on [^{3}H]ketanserin binding from bovine brain; while there was no statistical difference between the values obtained for the datum point from the two laboratories, it was considered to be a source of experimental variability. The controversy over whether the 5-HT_2 binding sites exists in different "states" or "subtypes" is considered to result from differences in the interpretation of data.

1.3.2.3 5-HT_3

The "M" receptor, first identified by Gaddum and Picarelli (1957) was designated "5-HT_3" in the classification proposed by Bradley *et al.* (1986). The "M" receptor was first identified in the periphery on the basis of the inability of methiothepin or ketanserin to block the excitatory effects of 5-HT. Soon selective antagonists were developed, and their effects on animal behaviour suggested an action in the CNS (Costall *et al.*, 1987). Kilpatrick *et al.* (1987) and Watling (1988), using tritiated versions of these antagonists, demonstrated the existence of the 5-HT_3 binding sites in a number of regions of the mammalian brain. The highest number of binding sites were found in the cortex and the limbic areas, including the nucleus accumbens. Based on the location of these sites it was proposed that agents selective for the 5-HT_3 site may possess anxiolytic and/or antipsychotic activity; the results of such studies, however, are inconclusive. For example, GR 38032F is reported (Jones *et al.*, 1987) to display anxiolytic activity in the social interaction test of anxiety but is inactive in the Vogel test. In contrast, File and Johnston (1989), did not observe 5-HT_3 antagonist compounds to display anxiolytic activity in either the social interaction test or the elevated plus-maze test, but they attributed the anxiolytic effects of GR 38032F, observed by (Jones *et al.*, 1987) to the low baseline scores of social interaction. Nevertheless, 5-HT_3 antagonists are potent anti-emetics (Andrews *et al.*, 1988), and GR 38032F (ondansetron) is currently being evaluated in man (Cubeddu *et al.*, 1990).

1.3.3 Discrepancies between radio-ligand binding studies and electrophysiologically characterized 5-HT receptors

The classical 5-HT antagonists which have high affinities for 5-HT_1 and 5-HT_2

receptors, fail to antagonize S_3-mediated responses. Methiothepin has a high affinity for 5-HT_1 and 5-HT_2 binding sites (Leysen *et al.*, 1981) but it fails to block S_1-mediated facial motorneurone responses (McCall and Aghajanian, 1980), suggesting that neither binding site corresponds to the S_1-receptor.

A possible reason for such discrepancies lies in the fact that [^{3}H]spiperone (when used to characterize 5-HT binding sites), has a thousand times greater affinity than the neurotransmitter itself (Peroutka and Snyder, 1979), and such high concentrations of 5-HT may have non specific effects. In addition, Leysen (1984), questioned the idea that the 5-HT_1 binding site corresponds to a 5-HT receptor on the grounds of its few functional correlates, and due to the limited number of chemical congeners. However, the development of more specific compounds, for example, 8-OH-DPAT (Arvidsson *et al.*, 1981), TVX Q 7821 (ipsapirone) (Traber *et al.*, 1984), and buspirone (Peroutka, 1985), refutes this possibility.

1.3.4 Biochemical evidence for multiple 5-HT receptors

1.3.4.1 5-HT_{1A} sites

5-HT stimulates adenylate cyclase in the rat (Markstein *et al.*, 1986) and guinea-pig (Shenker *et al.*, 1987) hippocampal membranes. The enhancement of adenylate cyclase in the rat was correlated with the affinity of several agonists for the 5-HT_{1A} binding site (Markstein *et al.*, 1986), whereas in the guinea-pig the stimulation of adenylate cyclase was mediated by two distinct 5-HT receptors: a high affinity receptor which appears to be a functional correlate of the 5-HT_{1A} binding site, and a low affinity receptor which does not correspond to a known 5-HT binding site (Shenker *et al.*, 1987).

De Vivo and Maayani (1985,1986) reported the presence of a 5-HT receptor which functions to inhibit forskolin-stimulated adenylate cyclase in the guinea-pig and rat hippocampal membranes. This inhibition was guanine triphosphate (GTP)-dependent in the guinea-pig and rat (De Vivo and Maayani, 1985), and pertussis toxin sensitive in the rat (Clarke *et al.*, 1987). The 5-HT_{1A} agents (5-CT, 8-OH-DPAT and buspirone) are potent agonists at this receptor, while methiothepin and spiperone are potent antagonists. Furthermore, the 5-HT_2 and the 5-HT_3 receptor antagonists, ketanserin and ICS 205 930 respectively, exhibit little antagonist activity at this site (De Vivo and Maayani, 1986).

1.3.4.2 5-HT_{1B} sites

Several studies report the presence of an adenylate cyclase system in serotonin nerve terminals. Forskolin is reported to enhance the stimulation-evoked release of 5-HT from rat brain cortex (Schoffelmeer *et al.*, 1985 ; Schlicker *et al.*, 1987) and hypothalamic slices (Galzin *et al.*, 1987) and cortical synaptosomes (Schlicker *et al.*, 1987), an effect that was mimicked by 8-Br-cAMP, but not potentiated by phosphodiesterase inhibitors. However, it was noted that the effects of 5-HT and methiothepin on 5-HT release remain unaltered in the presence of forskolin and a phosphodiesterase inhibitor (Schlicker *et al.*, 1987).

The inhibitory effect of 5-HT agonists on 5-HT release is abolished by the pretreatment of brain slices with pertussis toxin (Passarelli *et al.*, 1988), thus suggesting that a G protein is necessary for autoreceptor function. In addition, the facilitatory effect of methiothepin on the stimulation-evoked release of 5-HT is unaltered by pertussis toxin pretreatment (Passarelli *et al.*, 1988).

Bouhelal *et al.* (1988), using membranes of the substantia nigra (an area rich in the 5-HT_{1B} binding sites), reported several 5-HT agonists to inhibit forskolin-stimulated adenylate cyclase, whilst being ineffective on unstimulated adenylate cyclase. The 5-HT_{1A} agonists, 8-OH-DPAT and ipsapirone, exhibited little agonist activity at this site. Of the antagonists, spiperone, ketanserin and mesulergine were inactive at reversing the inhibitory effect of 5-HT on forskolin-stimulated adenylate cyclase, whereas cyanopindolol, propranolol and metergoline fully reversed its effect. Such a pharmacological profile suggests that this 5-HT receptor, negatively coupled to adenylate cyclase in the substantia nigra, resembles the 5-HT_{1B} binding site.

Phorbol esters that stimulate protein kinase C, increase the rate of 5-HT synthesis in rat hippocampal and hypothalamic synaptosomes (Fillenz and Pei, 1987). In addition, they potentiate the stimulation-evoked release of 5-HT from rat hypothalamic (Ramdine *et al.*, 1987) and cerebral cortex slices (Wang and Friedman, 1987), whereas, the protein kinase C inhibitor, exerts the opposite effect (Wang and Friedman, 1987). However, the inconsistency of the effect of compounds to interfere with agents acting as 5-HT autoreceptor agonists and antagonists, suggests that although protein kinase C can modulate the synthesis and release of 5-HT, it is not directly involved in the autoreceptor

transduction pathway (Wang and Friedman, 1987).

1.3.4.3 5-HT_{1D} sites

Although the 5-HT_{1D} binding site represents only a small proportion of total 5-HT_1 sites in the rat brain, its anatomical distribution in the bovine (Heuring and Peroutka, 1987), guinea-pig and human (Waeber *et al.*, 1989) brain resembles very closely that of the 5-HT_{1B} site in the rat brain (Pazos and Palacios, 1985 ; Waeber *et al.*, 1989). Schoeffter *et al.* (1988) investigated the secondary messenger system coupled to this site in the calf substantia nigra. These investigators demonstrated 5-HT and a number of 5-HT related compounds to inhibit the forskolin-stimulated adenylate cyclase, the inhibition being GTP-dependent. The rank order of potency of 15 compounds acting as agonists on this receptor was almost the same as that of their affinity for the 5-HT_{1D} binding site. In addition, 8-OH-DPAT, buspirone and ipsapirone exhibited weak agonist activity in inhibiting forskolin-stimulated adenylate cyclase. The inhibitory effect of 5-HT was antagonized by methiothepin, mianserin and spiperone yielding displacement values close to their affinity values at the 5-HT_{1D} binding site, suggesting that this 5-HT receptor, negatively coupled to adenylate cyclase in calf substantia nigra, belongs to the 5-HT_{1D} subtype. Moreover, Schoeffter and Hoyer (1989), based on the differences in the activity of agents, were able to distinguish between 5-HT_{1B} and 5-HT_{1D} receptors linked to adenylate cyclase in the rat and calf substantia nigra, respectively.

1.3.4.4 5-HT_{1C} and 5-HT_2 sites

The rat choroid plexus contains a high density of the 5-HT_{1C} binding sites (Pazos and Palacios, 1985), whereas the frontal cerebral cortex of the rat and guinea-pig contain the greatest number of the 5-HT_2 binding sites (Leysen *et al.*, 1982). These two binding sites are very similar, pharmacologically and possess several characteristics in common. For example, a number of agonists and antagonists display similar affinities for both these sites, their binding being G protein dependent (Hoyer,1988). In addition, Conn and Sanders-Bush (1987) demonstrated both these sites to be linked to the same second messenger system (*i.e.* phosphoinositide hydrolysis). Hartig (1989) described the cloning of the 5-HT_{1A}, 5-HT_{1C} and 5-HT_2 receptor and the subsequent determination of their

amino acid sequences. The 5-HT_{1C} and the 5-HT_2 receptor share about 80% amino acid sequence homology, suggesting a common ancestry, which may account for the similarity in drug affinities. They were however quite different from the 5-HT_{1A} receptor, with whom they share only 40% homology; based on this finding he proposed a molecular biological classification of 5-HT receptors into two superfamilies, namely the G protein receptor linked superfamily, and the ligand-gated ion channel superfamily.

1.3.4.5 5-HT_3 sites

Derkach *et al.* (1989) demonstrated the 5-HT_3 binding site in the guinea-pig submucous plexus to be associated with an ion channel permeable to cations. Application of 5-HT was associated with an inward current which was antagonized by 5-HT_3 antagonists. Also, responses were unaffected by pretreatment with pertussis toxin, suggesting that G proteins are not necessary for functioning.

1.3.4.6 5-HT_4 sites

A novel 5-HT receptor identified in guinea-pig hippocampal membranes and in mouse colliculi neurones (Dumuis *et al.*, 1988) was designated "5-HT_4" because it is pharmacologically dissimilar from the 5-HT receptors classified so far. This receptor is positively coupled to adenylate cyclase (Dumuis *et al.*, 1988). Its radio-ligand binding identification is not possible due to a lack of putative high affinity compounds for this site.

1.3.5 Behavioural models linked to 5-HT receptor activation

Several behaviours described in the literature are considered to have a 5-HT component. These include locomotor activity, thermoregulation, aggression, sexual behaviour, feeding, head-twitch/wet-dog shake and the 5-HT behavioural syndrome. It is beyond the scope of this section to cover these in detail, but the reader is referred to a recent review for further information (Glennon and Lucki, 1988).

1.4 Autoreceptors for 5-HT in the central nervous system

Many factors are involved in the control of neurotransmitter release. Indeed, receptors located presynaptically on cell bodies and nerve terminals seem to play a prominent role in the modulation of synaptic transmission both in the central and in the peripheral nervous system. Those receptors located on nerve terminals and activated by the neurotransmitter released from the same nerve terminal are termed "presynaptic- or terminal-autoreceptors", while receptors located on cell bodies and or dendrites activated in a similar manner are termed "somadendritic autoreceptors". Both of these receptors are considered to play an important role in regulating the activity of serotoninergic neurones. Although autoreceptors have been identified for a great number of neurotransmitters, this section will concentrate only on the information relating to the terminal and the somadendritic 5-HT autoreceptor, in the rat.

1.4.1 *in vitro* evidence for the existence of the terminal 5-HT autoreceptor

The terminal 5-HT autoreceptor has been identified in brain regions receiving either ascending or descending projections, originating from 5-HT cell bodies in the raphe. This 5-HT autoreceptor has been most extensively studied in the rat brain but is also found in the brains of other species (see Table 1).

Farnebo and Hamberger (1971) first demonstrated that the 5-HT receptor agonist, LSD (lysergic acid diethylamide), inhibited the electrically-evoked release of [^{3}H]5-HT from slices of rat cerebral cortex, whereas methiothepin (a putative 5-HT autoreceptor antagonist) was shown to increase the rate of release of [^{3}H]5-HT (Farnebo and Hamberger, 1974). In 1979, extracellularly-applied 5-HT was shown to inhibit the depolarization-evoked release of [^{3}H]5-HT from synaptosomes (Cerrito and Raiteri, 1979) and slices (Gothert and Weinheimer, 1979). The inhibitory effect of 5-HT was antagonized competitively by methiothepin, suggesting that the facilitatory effect of methiothepin was probably the result of its antagonism of the action of released 5-HT on its autoreceptor (Gothert, 1980).

Martin and Sanders-Bush (1982) were the first to suggest that the terminal 5-HT

Table 1.

Terminal 5-HT autoreceptor identification in the CNS of a number of species

Species	Brain region	Preparation	Reference
Rat	Cerebellum	Synaptosome	Bonanno *et al.*, 1986
	Cerebral cortex	Slice	Gothert and Weinheimer, 1979
			Middelmiss, 1984a,b
			Gothert *et al.*, 1987
	Hippocampus	Slice	Langer and Moret, 1982
			Richards, 1985
		Synaptosome	Cerrito and Raiteri, 1979
	Hypothalamus	Slice	Galzin *et al.*, 1985
	Raphe	Slice	Sawada and Nagatsu, 1986
			Middlemiss, 1987
	Spinal cord	Synaptosome	Monroe and Smith, 1985
		Slice	Murphy and Zemlan, 1988
	Striatum	Slice	Middlemiss, 1984b
Mouse	Cerebellum	Slice	Figueroa *et al.*, 1985
Rabbit	Cerebral cortex	Slice	Limberger *et al.*, 1986
	Hippocampus	Slice	Feuerstein *et al.*, 1987
	Hypothalamus	Slice	Schoups and De Potter, 1988
Guinea-pig	Cerebral cortex	Slice	Middlemiss *et al.*, 1988
Pig	Cerebral cortex	Slice and	Schlicker *et al.*, 1989
		Synaptosome	Fink *et al.*, 1988
Human	Cerebral cortex	Slice	Schlicker *et al.*, 1985b
			Galzin *et al.*, 1988a

autoreceptor resembles more closely the 5-HT_1 binding site. Further evidence in support of this hypothesis came from functional studies investigating the effects of a range of tryptaminergic agonists on the terminal 5-HT autoreceptor (Engel *et al.*, 1983 ; Gothert and Schlicker, 1983). In addition, the 5-HT_2 receptor antagonists, ketanserin and spiperone were without effect at this receptor (Engel *et al.*, 1983,1986). The 5-HT autoreceptor is unlikely to correspond to the 5-HT_3 binding site, because 2-methyl-5-HT exhibits weak agonist activity (Engel *et al.*, 1986), while the antagonist MDL 72222 is without effect (Middlemiss, personal communication).

A recent comprehensive study correlated the affinity of 17 5-HT agonists and 13 antagonists for 5-HT binding sites with their functional activity at the terminal 5-HT autoreceptor in the rat cortex (Engel *et al.*, 1986). A good correlation was obtained between their potencies or affinities for the 5-HT autoreceptor and their affinities at the 5-HT_{1A} and the 5-HT_{1B} binding sites. However, the best correlation was obtained with 5-HT_{1B} binding site, suggesting that the terminal 5-HT autoreceptor in rat cortex corresponds to the 5-HT_{1B} binding site. The authors endeavour to point out that the distinction between the 5-HT_{1A} and 5-HT_{1B} site would have been more prominent if the non-tryptamine derivatives, for example 8-OH-DPAT, ipsapirone and the 5-HT antagonists, spiperone and cyproheptadine, had been taken into account.

8-OH-DPAT and buspirone exhibit considerable affinity for the 5-HT_{1A} binding site (Middlemiss and Fozard, 1983 ; Peroutka, 1985), but were demonstrated to be inactive as agonists or antagonists at the terminal 5-HT autoreceptor in the frontal cortex of the rat (Midddlemiss, 1984b,1988), whereas Hamon *et al.* (1984) reported 8-OH-DPAT to attenuate the release of 5-HT from striatal slices, however at such a high concentration it may have acted at the 5-HT_{1B} receptor. In contrast, RU 24969 possesses a high affinity for the 5-HT_{1B} binding site (Sills *et al.*, 1984), and was a potent inhibitor of the release of 5-HT (Middlemiss, 1985a ; Gothert *et al.*, 1987). These findings add further credence to the idea that the terminal 5-HT autoreceptor does not correspond to the 5-HT_{1A} binding site, but instead bears similarity to the 5-HT_{1B} binding site.

To date, the terminal 5-HT autoreceptor has been identified in a variety of brain regions of a number of species (Table 1). Pharmacological analysis of this 5-HT

autoreceptor in a number of regions of rat brain indicates no major differences with respect to the activity of agonists and antagonists, suggesting that these regionally distributed autoreceptors are similar and probably conserved within species.

Notably, the 5-HT_{1B} binding site has only been identified in rat and mouse brain, but is absent in guinea-pig, human, pig, pigeon and a number of other species. Although a 5-HT_{1B} binding site could not be identified in these species, they were instead demonstrated to possess a 5-HT_{1D} binding site (Heuring *et al.*, 1986 ; Hoyer *et al.*, 1986a ; Martial *et al.*, 1989 ; Waeber *et al.*, 1989). Autoradiographic studies have shown the distribution of the 5-HT_{1D} site in guinea-pig and human brain (Waeber *et al.*, 1989) resembles the distribution of the 5-HT_{1B} site in rat brain (Pazos and Palacios, 1985 ; Waeber *et al.*, 1989).

Several research groups have identified a terminal 5-HT autoreceptor in guinea-pig, human, pig and rabbit brain (Feuerstein *et al.*, 1987 ; Middlemiss *et al.*, 1988 ; Galzin *et al.*, 1988b ; Schlicker *et al.*, 1989), which was different pharmacologically from the terminal 5-HT autoreceptor identified in the rat. This 5-HT autoreceptor in these species may bear similarity to the 5-HT_{1D} binding site (Hoyer *et al.*, 1987 ; Hoyer and Middlemiss, 1989).

Although the terminal 5-HT autoreceptor found in the rat and mouse brain is pharmacologically different from that found in several other mammals, in particular man, this difference in classification should not detract from the fact that they are both involved in regulating the release of 5-HT from serotoninergic nerve terminals.

1.4.2 Characterization of the 5-HT binding site on 5-HT terminals

Early studies using radio-ligand binding and autoradiography had been unable to demonstrate any changes in the number of 5-HT_1 binding sites in the cortex, hippocampus or the locus coeruleus after the destruction of serotoninergic nerve terminals with 5,7-DHT (Whitaker and Deakin, 1981 ; Verge *et al.*, 1985 ; Weissmann-Nanopoulos *et al.*, 1985). More recent studies, however, using quantitative autoradiography, reported a reduction in the number of 5-HT_1 binding sites in the dentate gyrus and the anterior

hippocampus following the intracerebroventricular administration of 5,7-DHT (Fischette *et al.*, 1987). Furthermore, destruction of the serotoninergic nerve bundle leaving the anterior raphe intact, using 5,7-DHT, was associated with a reduction in the number of non-5-HT_{1A} binding sites (presumably 5-HT_{1B}) in the substantia nigra (Verge *et al.*, 1986). These results suggest that at least some of the 5-HT_1 sites in the dentate gyrus, anterior hippocampus and part of the non-5-HT_{1A} in the substantia nigra, are probably located on serotoninergic nerve terminals and may correspond to the 5-HT_{1B} autoreceptor. The inability of some studies to detect changes, following the degeneration of serotoninergic neurones is probably due to the small numbers of terminal 5-HT autoreceptors as well as the timing of the of the studies following degeneration.

1.4.3 Mechanism of regulation of release

The inhibitory effect of 5-HT on the release of [^{3}H]5-HT is reported to be related inversely to the stimulation frequency applied or the concentration of Ca^{2+} in the extracellular fluid, analogous to that observed for central and peripheral noradrenergic neurones (Langer, 1977). Gothert (1980) proposed that the action of 5-HT autoreceptor agonists and antagonists to control 5-HT release was the consequence of their ability to alter the affinity of the voltage-sensitive permeability channels of the cell membrane for Ca^{2+} ions, thus modulating the amount available for stimulus release coupling.

The influx of Ca^{2+} ions is involved in the initiation, but several recently identified intraneuronal components have been implicated in mediating molecular events which bring about neurotransmitter release. Two of the more prominent candidates so far identified are synaptophysin and synapsin I (Trimble and Scheller, 1988). Synaptophysin, is located in the synaptic membrane, with its cytoplasmic domain possessing a Ca^{2+} binding site. The binding of Ca^{2+} ions to this site of synaptophysin is considered to play a role in the exocytosis of neurotransmitter from synaptic vesicles. In contrast, synapsin I is found in the cytoplasm of all neurones, where it binds with high affinity to synaptic vesicles and to cytoskeletal components *e.g.* microtubules. This protein is rapidly phosphorylated during depolarization by cAMP- and Ca^{2+}/calmodulin-dependent protein kinases, leading to its dissociation from synaptic vesicles, thus enabling release of vesicular contents.

These two vesicle associated proteins represent only a small fraction of the components so far identified and implicated in regulating neurotransmitter release. Future research in this field is likely to lead to a better understanding of the molecular events as well as the mechanism/s by which presynaptic receptors function to modulate neurotransmitter release.

1.4.4 Compounds active at the terminal 5-HT autoreceptor

In the rat, [^{3}H]5-HT release is inhibited by 5-HT receptor agonists, all of which incorporate the indole nucleus. The most potent of these is 5-CT, pIC_{50}=7.65 (Engel *et al.*, 1986) followed by RU 24969, pIC_{50}=7.45 (Middlemiss, 1985a), the natural agonist 5-HT, pIC_{50}=7.34 (Middlemiss, 1984b) and LSD pIC_{50}=5.50 (Engel *et al.*, 1986).

Antagonists in terms of decreasing potency are: (-)cyanopindolol, pA_2=8.30 (Schlicker *et al.*, 1985a); (isopropylester) of (±)-4[3-ter-butyl-amino-2-hydroxypropoxy]indol-2-carbonic acid [(±)-21-009], pA_2=7.49 (Engel *et al.*, 1986); (-) methiothepin, pA_2=6.81 (Hibert and Middlemiss, 1986); (-)propranolol, pA_2=6.72 (Middlemiss, 1984a); (-)pindolol, pA_2=6.57 (Engel *et al.*, 1986) and quipazine, pA_2=5.29 (Engel *et al.*, 1983). Some of these compounds are competitive antagonists (Gothert, 1980 ; Middlemiss, 1986), and some are also indole derivatives. Interestingly, with the exception of quipazine (which does not possess a chiral centre), the antagonist activity of the other compounds resides primarily in their *laevo* enantiomer (Nahorski and Willcocks, 1983 ; Middlemiss, 1984a,1986 ; Schlicker *et al.*, 1985a ; Hibert and Middlemiss, 1986). Methiothepin has been shown to increase the release of endogenous 5-HT from rat cortical and hypothalamic slices (Pettibone and Pflueger, 1984). In addition, some of these autoreceptor antagonists facilitate the stimulation-evoked release of 5-HT, when applied to brain slices. This is generally thought to be due to their antagonism of the endogenous tone created by the release of endogenous 5-HT on the presynaptic autoreceptor. However, some *beta*-adrenoceptor antagonists, at doses which exhibit antagonist activity at the autoreceptor, do not facilitate the stimulation-evoked release (Middlemiss, 1984a,1986). This is considered to be due to their partial agonist activity profile (Hjorth and Carlsson,

1986 ; Maura *et al.*, 1987), and separate from their membrane stabilizing activity (Barrett and Cullum, 1968).

Several of these autoreceptor antagonists are also potent *beta*-adrenoceptor antagonists, their antagonist activity at the 5-HT autoreceptor is unlikely to be mediated by a particular subtype of *beta*-receptor, because of the lack of effect of the *beta*-adrenoceptor agonists, isoprenaline (Schlicker *et al.*, 1985a) and salbutamol (Baumann and Waldmeier, 1981) and the $beta_1$- and $beta_2$-selective antagonists, atenolol and ICI 118551, respectively (Middlemiss, 1986).

As for the terminal 5-HT_{1D} autoreceptor, the rank order of potency of agonists and antagonists is different to that found at the rat 5-HT_{1B} autoreceptor. The rank order of potency of agonists and antagonists at the terminal 5-HT autoreceptor is given below for a number of species :

(1) Guinea-pig cerebral cortex slices (Middlemiss *et al.*, 1988).

agonists : 5-CT (pIC_{50}=8.1) > 5-HT (pIC_{50}=7.4) > RU 24969 > GR 43175 ;

antagonists : methiothepin (pA_2=8.2) > metergoline > methysergide = cyanopindolol (pA_2= 6.5) = yohimbine > mesulergine ;

(2) Pig cerebral cortex slices (Schlicker *et al.*, 1989).

agonists : 5-HT > 5-methoxy-N,N-dimethyl-tryptamine = 5-CT > RU 24969 > yohimbine > cyanopindolol > 8-OH-DPAT ;

antagonists : methiothepin > metergoline > mianserin ;

(3) Rabbit hippocampal slices (Feuerstein *et al.*, 1987).

agonists : 5-CT > 5-HT > 5-methoxy-N,N-dimethyl-tryptamine > 8-OH-DPAT > methysergide > cyanopindolol ;

antagonists : methiothepin > metergoline > cyanopindolol ;

(4) Human cerebral cortex slices (Galzin *et al.*, 1988a,b).

agonists : 5-CT > RU 24969 ;

antagonists : methiothepin > metergoline = methysergide > propranolol.

It is worth noting that *beta*-adrenoceptor antagonists are only weakly active at the terminal 5-HT_{1D} autoreceptor in contrast to their marked potency at the 5-HT_{1B} autoreceptor. Furthermore, methiothepin is 24-40 times more potent at the guinea-pig 5-

HT_{1D} as compared to the rat 5-HT_{1B} autoreceptor (Middlemiss *et al.*, 1988).

1.4.5 Heteroreceptors regulating 5-HT release

In addition to possessing 5-HT autoreceptors, 5-HT nerve terminals are also endowed with a host of other receptors for other neurotransmitters. These receptors are known as presynaptic heteroreceptors, they are not activated by the 5-HT released form nerve terminals but can function to modulate its release. These heteroreceptors are generally studied using *in vitro* superfusion techniques. To date, a multitude of different heteroreceptors have been identified and their respective subtypes characterized. The most extensively studied of these is the *alpha*$_2$-adrenoceptor in the cerebral cortex (Gothert and Huth, 1980) and the hippocampus (Frankhuijzen and Mulder, 1980 ; Maura *et al.*, 1982). Neurotransmitter receptors located on 5-HT nerve terminals, which can also modulate 5-HT release include nicotinic and muscarinic (Marchi *et al.*, 1986), $GABA_B$ (Schlicker *et al.*, 1984), histamine H_3 (Schlicker *et al.*, 1988) and dopamine D_2 (Drescher and Hetey, 1988) receptors.

As these heteroreceptors are able to modulate 5-HT release, similarly, 5-HT heteroreceptors can also exercise control on the release of several other neurotransmitters in the brain. These 5-HT heteroreceptors are involved in regulating the release of acetylcholine (Maura *et al.*, 1989), dopamine (Ennis *et al.*, 1981) and noradrenaline (Molderings *et al.*, 1989). The subtype of the 5-HT receptor involved in these effects has only been determined for acetylcholine release from slices of rat hippocampus, where it was reported to be of the 5-HT_{1B} subtype (Maura *et al.*, 1989). Furthermore, the 5-HT receptor located on sympathetic nerves of the rat vena cava is also of the 5-HT_{1B} subtype (Molderings *et al.*, 1987).

1.4.6 Interaction between the terminal 5-HT autoreceptor and the neuronal uptake site for 5-HT

In rat hypothalamic slices, the electrically-evoked release of [^{3}H]5-HT is inhibited in a dose-dependent manner by LSD. This inhibitory effect of LSD is attenuated by

methiothepin and by the 5-HT uptake blocker, citalopram (Langer and Moret, 1982). This activity of citalopram was reproduced by a range of structurally diverse 5-HT uptake inhibitors, and was unaffected by PCPA-depletion, suggesting that it was unrelated to the concentrations of 5-HT in the synaptic cleft (Galzin *et al.*, 1985). On this basis, these authors proposed that 5-HT uptake inhibitors and 5-HT autoreceptor agonists interact by some functional link between the uptake mechanism and the autoreceptor, leading to a reduced autoreceptor function, similar to that proposed for noradrenergic neurones (Pelayo *et al.*, 1980). Further extension of these studies demonstrated that this interaction occurred only under conditions of electrical stimulation (Galzin *et al.*, 1986 ; Passarelli *et al.*, 1987).

Bonanno and Raiteri (1987) also found citalopram attenuated the effect of 5-HT autoreceptor agonists on the stimulation-evoked release of [^{3}H]5-HT from rat cerebral cortex slices but not synaptosomes. Their findings suggested that the attenuating effect of citalopram was independent of the method of stimulation, occurring equally well under electrical as well as potassium-evoked release. They also questioned the existence of a functional link between the 5-HT autoreceptor and the 5-HT neuronal uptake site, instead attributing the effect of citalopram to the increased concentration of 5-HT expected in the synaptic cleft following 5-HT re-uptake block.

As regards to 5-HT neurones, if a functional link is present, then it may be of relevance in the treatment of depression because many antidepressant drugs are potent inhibitors of the neuronal uptake of 5-HT. Galzin *et al.* (1985) proposed that clinically prescribed antidepressant drugs, which block the neuronal uptake of 5-HT, may also lead to an attenuation of the activation of the 5-HT autoreceptor resulting in the enhanced release of 5-HT.

As yet, it is not clear whether a functional link exists between the 5-HT autoreceptor and the 5-HT neuronal uptake site or whether the uptake inhibitors influence the autoregulation by increasing synaptic concentrations of 5-HT.

1.4.7 *In vitro* identification of the somadendritic 5-HT autoreceptor

In vitro brain slices containing the dorsal raphe nucleus are monitored

electrophysiologically for their spontaneous firing activity. The spontaneous firing rate in slices, *in vitro*, is reported to be lower than that found *in vivo*, this is thought to result from the removal of an excitatory noradrenergic input (Vander Maelen and Agahajanian, 1983). In contrast, Trulson and Crisp (1986) have reported the firing rate of 5-HT neurones to be higher *in vitro*. This discrepancy may be due to the membrane stabilizing action of chloral hydrate in the study of Vander Maelen and Agahajanian (1983).

In general, 5-HT, 5-HT_{1A} agonists and compounds that enhance 5-HT function, depressed the firing rate of these neurones, by an action on the 5-HT autoreceptor, while the depletion of 5-HT does not alter spontaneous firing rate (Trulson and Arasteh, 1986 ; Sprouse and Aghajanian, 1987 ; Trulson and Crisp, 1986). The 5-HT receptor antagonists, methiothepin, cyproheptadine and methysergide, did not alter the spontaneous firing rate, although methiothepin attenuated the inhibitory effect of 5-HT (Trulson and Crisp, 1986). In addition, the inhibitory effect of the putative 5-HT_{1A} receptor agonist, CM 57493, was antagonized by propranolol (Adrien *et al.*, 1989). These *in vitro* studies should be interpreted with caution since rather high concentrations of agonists and antagonists were used.

1.4.8 *In vivo* evidence for the existence of the terminal 5-HT autoreceptor

Gallager and Aghajanian (1975) measured the *in vivo* release of newly-synthesized [^{3}H]5-HT form [^{3}H]tryptophan in ventricular perfusates of the rat. They observed that systemically administered LSD (75 or 150 µg/kg) totally inhibited raphe cell firing and also produced a reduction in [^{3}H]5-HT release. Although neuronal firing recovered to control rates 90 minutes after LSD administration, release was still depressed at this time. It was proposed that the inhibitory effect of LSD on neuronal firing was probably the result of its action on 5-HT cell bodies, yet it was unable to account completely for the reduction in [^{3}H]5-HT release from terminals. In 1979, Hery *et al.*, using a similar perfusion technique to Gallager and Aghajanian (1975), and measuring [^{3}H]5-HT release from the caudate nucleus of encéphale isolé cats, demonstrated that locally applied LSD inhibited the release evoked by elevated potassium ions. These findings suggest that LSD also has an effect on 5-HT receptors located on presynaptic 5-HT terminals.

Chaput *et al.* (1986a) measured the effect of electrical activation of the ascending 5-HT pathway on the firing activity of postsynaptic hippocampal pyramidal neurones before and after the systemic administration of methiothepin. Methiothepin markedly increased the duration of the suppression of firing activity of these neurones without altering their responsiveness to microiontophoretically applied 5-HT. These *in vivo* findings suggest that methiothepin enhances synaptic transmission by blocking the activation of the terminal 5-HT autoreceptor by endogenously released 5-HT.

The recent development of intracerebral dialysis and *in vivo* voltammetry which are used to measure extracellular concentrations of 5-HT and its primary metabolite 5-HIAA respectively, have allowed the determination of drug effects on central 5-HT function. Martin and Marsden (1985) reported similar results using both these techniques. Microdialysis has allowed the estimation of the extracellular concentration of 5-HT, which in the frontal cortex of freely moving rats was reported to be about 0.1 μM (Maidment *et al.*, 1986). However, following the recent development of an *in vivo* voltammetric electrode which selectively measures 5-HT concentrations in anesthetized rat frontal cortex and dorsal raphe nuclei were reported to be about 5 and 10 nM respectively (Crespi *et al.*, 1988). This disagreement is probably due to the underestimation of actual concentrations in anesthetized animals (Maidment *et al.*, 1986), also, extracellular concentrations of 5-HT are not static but are constantly fluctuating, making their accurate estimation rather difficult. It is likely that the actual extracellular concentration in the frontal cortex is within the range used by Middlemiss (1984a,b) in *in vitro* studies to inhibit the evoked release of previously taken up [^{3}H]5-HT. It has been suggested that the terminal 5-HT autoreceptor is under tonic inhibitory control. Evidence in support of this hypothesis has been provided by *in vivo* voltammetry, where the systemic administration of the putative terminal 5-HT autoreceptor antagonist, methiothepin was shown to increase 5-HIAA concentrations in the hippocampus, hypothalamus, striatum (Baumann and Waldmeier, 1984) and SCN (Marsden and Martin, 1985a) of the rat. In contrast, the same dose of methiothepin reduced 5-HT release in the hippocampus as measured by microdialysis (Sharp *et al.*, 1989b), this effect was considered to result from its partial

agonist activity at the somadendritic 5-HT_{1A} autoreceptor.

Following systemic administration, the putative 5-HT_{1B} receptor agonist, RU 24969, decreases extracellular concentrations of 5-HIAA in the rat frontal cortex (Brazell *et al.*, 1985), SCN (Marsden and Martin, 1985a) and hippocampus (Sharp *et al.*, 1989a). In addition, a similar effect was seen upon local application of RU 24969 to the SCN but not after its direct application to the dorsal raphe nucleus (Marsden and Martin, 1985a). Also, the local application of RU 24969 to the hippocampus *via* the perfusion fluid was associated with a decrease in 5-HT release (Sharp *et al.*, 1989a, unpublished observations). Furthermore, the direct infusion of RU 24969 into the SCN prevents the dorsal raphe stimulated increase in 5-HIAA concentrations (Martin and Marsden, 1987). These results strongly suggest that the 5-HT_{1B} receptor regulating the release and metabolism of 5-HT is located in terminal containing regions and not on cell bodies or dendrites.

1.4.9 *In vivo* evidence for the existence of the somadendritic 5-HT autoreceptor

The systemic administration of 8-OH-DPAT or ipsapirone produces a decrease in extracellular 5-HIAA concentrations in the SCN (implying a decrease in 5-HT release and metabolism), this effect is antagonized by ipsapirone and the *alpha*$_2$-adrenergic antagonist, idazoxan (Marsden and Martin, 1986). A decrease in 5-HT release is also seen in the rat hippocampus, following the systemic administration of a number of 5-HT_{1A} agonists, using brain microdialysis (Sharp *et al.*, 1989a), whereas 5-HIAA concentrations were only slightly reduced or unchanged, suggesting that 5-HIAA may not be a good indicator of 5-HT released. In the same study, the common metabolite of buspirone, gepirone and ipsapirone, 1-(2-pyrimidinyl)-piperazine, which also exhibits some affinity for *alpha*$_2$-adrenergic binding site, was without effect on 5-HT release, implying that the action of its parent compounds and 8-OH-DPAT is likely to be mediated by the 5-HT_{1A} and not *alpha*$_2$-adrenoceptor. The poor correlation of 5-HIAA concentrations with 5-HT released, reported in this study, is likely to be the result of the inclusion of an uptake blocker in the perfusion fluid, which would be expected to interfere with the reuptake and the subsequent metabolism of 5-HT, however, an uptake blocker is often necessary in order

to achieve a stable and detectable concentration of 5-HT. In addition, changes in 5-HIAA concentrations are considered to be a reflection of the dose of 8-OH-DPAT used, as a low dose (10 μg/kg) was shown recently to inhibit firing and release, while having little effect on 5-HIAA concentrations in the rat frontal cortex (Garratt *et al.*, 1988 ; Crespi *et al.*, 1990). It therefore appears that the measurement of 5-HT metabolites may not provide an accurate reflection of alterations in the firing or the release of 5-HT.

The microiontophoretic application of 5-HT, 8-OH-DPAT and other 5-HT_{1A} agonists to the dorsal raphe nucleus produces a potent and consistent inhibition in raphe cell firing similar in magnitude to that seen following the systemic administration of these agents (Sprouse and Aghajanian, 1986,1987 ; Vander Maelen *et al.*, 1986 ; Blier and deMontigny, 1987); this inhibitory effect on firing is likely to be responsible for the reduction in 5-HT synthesis as measured by 5-HTP accumulation in a number of forebrain regions (Hjorth and Magnusson, 1988) as well as the decrease in 5-HT release in the hippocampus *in vivo* (Sharp *et al.*, 1989c). Furthermore, 8-OH-DPAT had no inhibitory effect on 5-HT release when applied to 5-HT terminal containing regions *in vitro* (Middlemiss, 1984b) or when added to the perfusion fluid during microdialysis, *in vivo* (unpublished observations reported by Sharp *et al.*, 1989b).

The inhibitory effect of 5-HT and 5-HT_{1A} agonists on raphe cell firing is considered to be the consequence of membrane hyperpolarization and decreased neuronal input resistance caused by an increase in membrane conductance to potassium ions (Sprouse and Aghajanian, 1987).

Several antagonists have been used to try to characterize the receptor/s involved in the regulation of 5-HT neuronal firing and release, following the administration of 5-HT agonists. Methiothepin, methysergide and metergoline display little antagonist activity to this response, (Haigler and Aghajanian, 1977 ; Steinberg *et al.*, 1987 ; Sharp *et al.*, 1989b), whereas, in electrophysiological studies, propranolol when administered directly to the dorsal raphe nucleus, antagonized the effect of ipsapirone (Sprouse and Aghajanian, 1986), but was only weakly active when administered systemically (Adrien *et al.*, 1989). This discrepancy may be due to the mode of administration, as Sharp *et al.* (1989b) also found propranolol to be inactive in attenuating the inhibitory effect of 8-OH-DPAT on the

release of 5-HT in the hippocampus when administered systemically. In contrast, pindolol partially attenuates the inhibitory effect of 8-OH-DPAT (Sharp *et al.*, 1989b), whereas, systemically administered spiperone attenuates the *in vivo* effects of 8-OH-DPAT and buspirone, on dorsal raphe firing (Lum and Piercey, 1988). Furthermore, the effect of 8-OH-DPAT is unlikely to be mediated by 5-HT_2, 5-HT_3, *alpha*$_{1/2}$-adrenoceptors or dopamine$_2$ receptors, as it proved resistant to antagonism by ritanserin, BRL 43694, phentolamine or sulpiride, respectively (Sharp *et al.*, 1989b).

Systemic administration or the direct infusion of low doses of 8-OH-DPAT into the raphe, produces hyperphagia in satiated rats. This hyperphagic response occurs independently of the behavioural syndrome, and is prone to antagonism by spiperone and pindolol, suggesting that it is likely to be mediated by the activation of the somadendritic 5-HT_{1A} receptor located in the raphe (Hutson *et al.*,1987).

Autoradiographic studies have identified a high density of the 5-HT_{1A} binding site on 5-HT cell bodies and or dendrites in the dorsal and median raphe region of the rat brain (Verge *et al.*, 1985 ; Weissmann-Nanopoulos, 1985 ; Thor *et al.*, 1990). The selective destruction of 5-HT cell bodies, using 5,7-DHT, was associated with a more pronounced reduction in the number of 5-HT_1 binding sites in the dorsal as compared to median raphe (Fischette *et al.*, 1987). In addition, a recent study has shown the dorsal raphe to be more responsive to 5-HT_{1A} agonists as compared to the median raphe (Blier *et al.*, 1990). Taken together, these results strongly suggest that 8-OH-DPAT, at low doses, preferentially activates the somadendritic 5-HT_{1A} autoreceptor at the level of the dorsal raphe, regulating 5-HT neuronal firing as well as the synthesis and release of 5-HT (Hjorth and Magnusson, 1988).

1.4.10 Functional adaptations of the 5-HT autoreceptor

The putative 5-HT autoreceptor antagonists augment the release of 5-HT from 5-HT neuroterminals both *in vitro* (Gothert, 1980 ; Schlicker *et al.*, 1985a) and *in vivo* (Baumann and Waldmeier, 1984 ; Chaput *et al.*, 1986a) are consistent with the presence of an endogenous tonic inhibitory 5-HT tone. This 5-HT autoreceptor lends itself to

functional studies of adaptation of receptor sensitivity. For example, the terminal 5-HT autoreceptor is desensitized by the chronic long-term stimulation induced by a selective 5-HT uptake inhibitor, in conjunction with an MAOI (Maura and Raiteri, 1984) or following chronic tricyclic antidepressant administration (Schoups and De Potter, 1988). Conversely, chronic blockade with the 5-HT antagonist, methiothepin, is reported to have mixed effects, it produced hypersensitivity of cortical 5-HT autoreceptors (Maura and Raiteri, 1984) but was without effect on hypothalamic 5-HT autoreceptors (Hagan and Hughes, 1983).

Electrophysiological studies, *in vivo*, report chronic administration of the 5-HT uptake inhibitors, citalopram (Chaput *et al.*, 1986b) or fluoxetine, but not the MAOI, clorgyline (Blier *et al.*, 1988), to desensitize the terminal 5-HT autoreceptor. In contrast, Sleight *et al.* (1989) using *in vivo* brain microdialysis, did not observe any changes in the release of 5-HT as modulated by the 5-HT autoreceptor, following the chronic administration of either amitriptyline or MDL 72394 (an MAOI).

The somadendritic 5-HT_{1A} autoreceptor is also desensitized following the chronic long-term administration of 5-HT uptake inhibitors (DeMontigny *et al.*, 1984 ; Chaput *et al.*, 1986b), an MAOI (Blier and deMontigny, 1985), 5-HT_{1A} agonist (Blier and deMontigny, 1987), and also after the partial depletion of 5-HT in the raphe (Chaput *et al.*, 1987). In contrast, the repeated administration of 8-OH-DPAT was recently demonstrated to be ineffective in altering the sensitivity of the somadendritic 5-HT_{1A} autoreceptor (Larsson *et al.*, 1990).

1.4.11 Therapeutic possibilities of compounds selective for the 5-HT autoreceptor

In the rat, the 5-HT_{1B} binding site has been correlated with the terminal 5-HT autoreceptor (Engel *et al.*, 1986). However, this 5-HT_{1B} binding site is only found in rat and mouse brain, and other species, namely, the human brain, do not possess a 5-HT_{1B} binding site, but instead possess a 5-HT_{1D} binding site (Heuring *et al.*, 1986 ; Hoyer *et al.*, 1986a). The terminal 5-HT autoreceptor in these species is considered to correspond to this 5-HT_{1D} binding site (Galzin *et al.*, 1988a ; Waeber *et al.*, 1989). Although these

two terminal 5-HT autoreceptors can be differentiated pharmacologically, they are functionally very similar (Hoyer and Middlemiss, 1989 ; Schoeffter and Hoyer, 1989).

The somadendritic 5-HT autoreceptor in rat brain is of the 5-HT_{1A} subtype (See section 1.4.9). Human brain contains a similar binding site in the raphe region, and this may correspond to the somadendritic 5-HT autoreceptor, which is conserved between species (Hoyer *et al.*, 1986a ; Palacios *et al.*, 1987).

The selective activation by agonists of the somadendritic 5-HT_{1A} autoreceptor located on 5-HT cell bodies is associated with an inhibition of neuronal firing and a decrease in 5-HT synthesis and release both locally and in terminal-containing regions (Hjorth and Magnusson, 1988). A similar effect on release and possibly synthesis of 5-HT in terminal-containing regions could be achieved by a selective terminal 5-HT autoreceptor agonist (Middlemiss, 1988). Thus, the selective activation of either autoreceptor would serve to decrease 5-HT function.

The development of a selective 5-HT autoreceptor agonist may have anxiolytic activity, since anxiolytic drugs, such as benzodiazepines (Stein *et al.*, 1975) and 5-HT_2 antagonists (Ceulemans *et al.*, 1985), are associated with a reduced 5-HT function. In support of this hypothesis the 5-HT_{1A} receptor partial agonist, buspirone has recently been introduced clinically (Goa and Ward, 1986). For a more extensive account of the anxiolytic potential of 5-HT selective compounds, the reader is referred to a review by Marsden (1990).

An antihypertensive role is forecast, based on the ability of urapidil to reduce blood pressure by a selective action on the somadendritic 5-HT_{1A} receptors located in the medulla (Mandal *et al.*, 1989). In addition, presynaptic 5-HT heteroreceptors belonging to the 5-HT_{1B} subtype have been identified on sympathetic nerves of rat vena cava (Gothert, 1988); their selective activation may also contribute to an antihypertensive effect.

Selective antagonists at the 5-HT autoreceptor would be expected to increase the release of 5-HT and thereby enhance 5-HT function. Currently available drugs that enhance 5-HT function are, the 5-HT uptake inhibitors (fluvoxamine and fluoxetine) used as antidepressants and the 5-HT releaser (fenfluramine) as an appetite suppressant. An enhanced 5-HT function may be involved in the the processing of noxious sensory stimuli in

the spinal cord, which suggests for a analgesic role (Hammond, 1985).

1.4.12 Aims of this study

A circadian rhythm in the concentration of 5-HT in rodent brain is well established, although the phase of the rhythm is not the same in all brain regions, the peak concentrations generally occur during the light phase. The observed rhythm appears to the the result of several factors ; thus, whereas the concentrations are higher during the light phase, the rate of synthesis and release are greatest during the dark phase, when the animals are most active. Release of 5-HT is, in turn, affected by many factors, including the activity of the terminal 5-HT autoreceptor which, when stimulated by 5-HT, inhibit transmitter release.

Previous studies have demonstrated a marked circadian variation in behavioural responses to 5-HT_2 receptor stimulation, while those associated with 5-HT_1 receptors are characterized by the apparent absence of any variation (Moser and Redfern, 1985).

Martin *et al.* (1987) proposed that the circadian variation rhythm in the release of 5-HT from central serotoninergic neurones derives, at least in part, from the corresponding rhythm in sensitivity of the terminal 5-HT_{1B} autoreceptor.

The aims of this study were therefore :

1) to gauge the functional activity of the terminal 5-HT autoreceptor over 24-hours, in order to determine whether it is directly responsible for generating or controlling the circadian rhythm in the release of 5-HT;

2) to examine the binding of [^{3}H]5-HT and relate this to the functional activity of the terminal 5-HT autoreceptor;

3) to determine the 24-hour activity of the *alpha*$_2$-adrenoceptors located on serotoninergic nerve terminals, as they can also modulate 5-HT release;

4) to monitor [^{3}H]ketanserin binding over 24-hours and relate this to the reported circadian variation in behaviour observed following 5-HT_2 receptor stimulation;

5) to measure the *in vivo* functional activity of the somadendritic 5-HT_{1A} autoreceptor, over 24-hours, since this receptor is able to exercise control over the synthesis and release of 5-HT.

2 NEUROTRANSMITTER RELEASE FROM BRAIN SLICES

2.1 HOUSING OF ANIMALS

2.1.1 Rats

Special cabinets were constructed to house the animals. Domestic extractor fans (Phillips, type HR 3408) were fitted to supply a constant current of air through the cabinets. One fan served six cabinets. Lighting (650-850 Lux) was supplied in each cabinet by a small white fluorescent tube (Phillips, TL 8W), with the choke removed and refitted outside the cabinet to prevent overheating. Each tube light was connected to a time switch (Smiths, TS 100 C). The inside of the boxes was lined with polystyrene (2.4 cm thick) to provide sound insulation. Plastic draught excluder foam strips (1.3 cm thick) were glued to the edge of the cabinet door to ensure light proofing. Similar cabinets have earlier been characterised and found to be adequate (Hillier, Davies and Redfern, 1973).

A single cage containing six rats was placed inside each cabinet. These animals were maintained on a 12 hour light : 12 hour dark cycle for at least 14 days prior to their use in experiments. Most of the experiments were performed during the normal working hours in the laboratory, however some were also performed outside normal working hours necessitating some animals to be phase shifted. These phase shifted animals were also allowed 14 days to entrain to the new light : dark cycle, prior to their use (Davies, Navaratnam and Redfern, 1974). When the animals were killed or their cages cleaned in the dark phase, the holding room was illuminated only by a low intensity red lamp (GEC, 15 W bulb, 4-6 Lux) since it has been shown not to have any significant effect on circadian rhythms (McGuire, Rand and Wurtman, 1973). The temperature of the room varied from 18-22°C, while the temperature inside the boxes varied from 20-23°C.

The animals had free access to both food (CRM pellets, Labsure) and water so that the animals could be left undisturbed for several days. However, regular inspections were made to ensure that the timers were working properly and that the animals had not managed to escape from the cages. Cages were cleaned every two to three days, with the cleaning times being randomized in order to prevent them from being interpreted as a cue. These conditions ensured that the animals were isolated from undue enviromental influences.

Male Wistar rats (University of Bath strain) weighing between 100-140g were housed in groups of six, with one group per cabinet (light on, 09.00 to 21.00 ; light off, 21.00 to 09.00). Some animals were phase shifted to the opposite of this lighting schedule. After 14 days of acclimatization the animals weighed between 200-350g.

2.1.2 Guinea-pigs

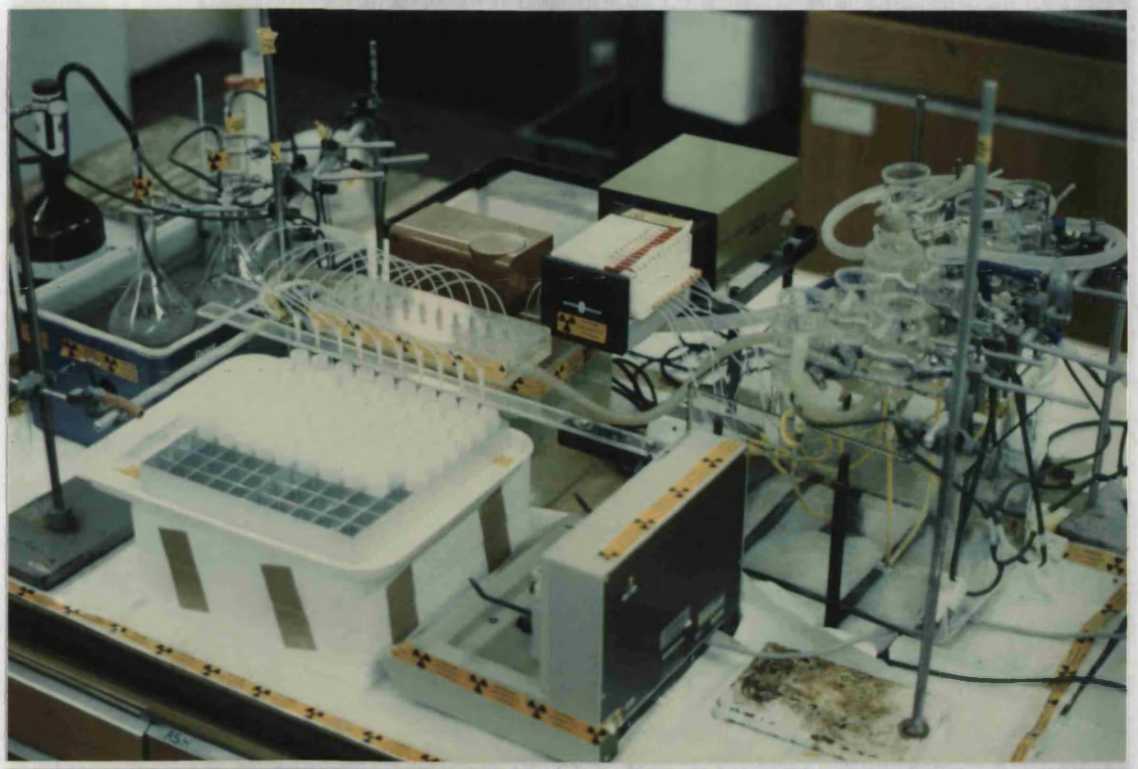
Guinea-pigs (Dunkin Hartley strain) of both sexes were used in these experiments. They were housed in groups of six, in stainless steel floor pens, each pen containing a reservoir of food (Labsure FD 1 pellets), and two water bottles. The animals also received a small supply of greens and hay. Two pens were placed in an air-conditioned room (temp. 18-22°C). The room was lit by two fluorescent tube lights (each 6 foot long) located on the ceiling. The lighting was controlled by a 24-hour timer located in a different room (light on, 09.00 to 21.00 ; light off, 21.00 to 09.00). The animals were kept on this lighting schedule for a minimum of two weeks prior to their use in experiments. When used they weighed between 450-550g. None of these animals were phase shifted.

2.2 SUPERFUSION STUDIES

2.2.1 The Superfusion System for tissue slices

The superfusion apparatus consisted of jacketed glass general tissue baths (150 ml capacity, used as reservoirs), linked *via* tubing to a pump which in turn supplied the superfusion chambers (Fig. 1 and Fig. 2). There were seven tissue baths which functioned to maintain buffer at 37°C and ensure that it is was continuously gassed with 95% O_2 and 5% CO_2. The outlet at the base of these baths was connected *via* rubber tubing to a three way tap, which allowed for the switching of buffers and reduced the possibility of introducing air into the tubes. Air is known to produce irregular release rates and at worst tissue death (Middlemiss, personal communication). The taps were in turn connected *via* silicone manifold tubing (Watson and Marlow, model TE 6), to a twelve cassette peristaltic pump head (Watson and Marlow, model 501 AA) operated by a Watson and Marlow pump (model 502 S). Each cassette was linked to the base of an individual chamber *via* silicone manifold tubing, the tubing first having to pass through a water bath

The Superfusion Apparatus



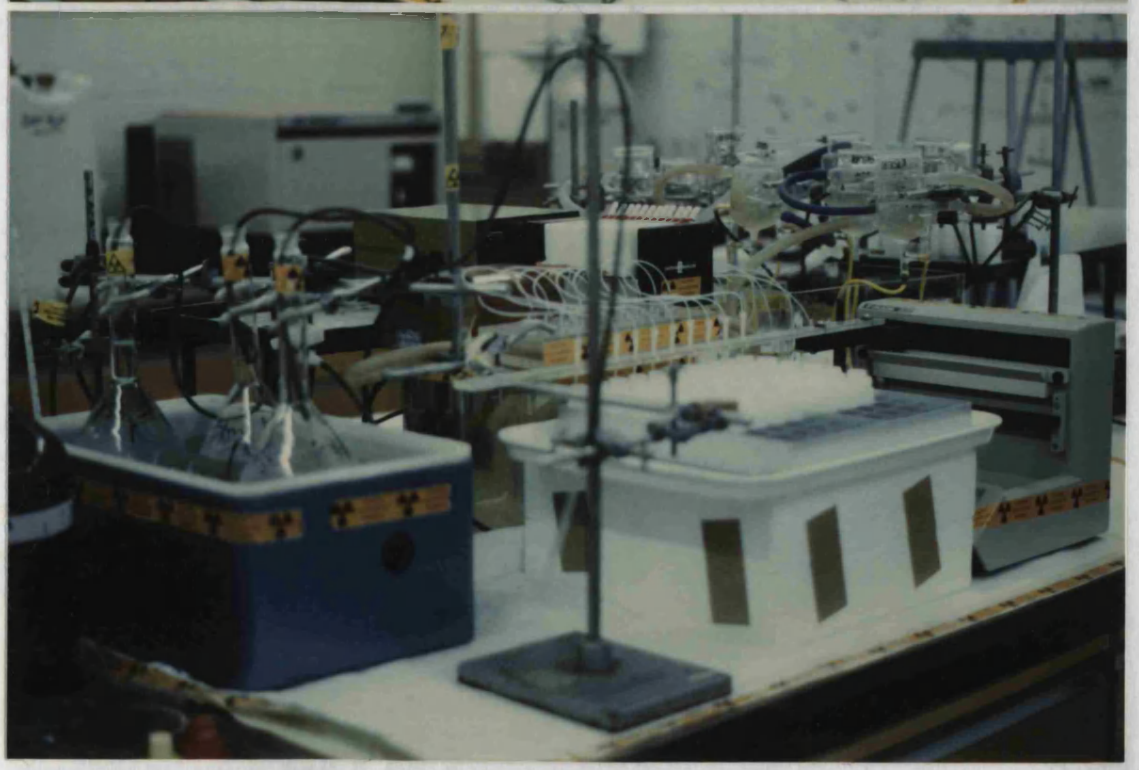


Figure 1. (top) and **Figure 2.** (bottom) These figures display the relative positioning of the various components comprising the superfusion system. These figures show the tissue baths (buffer reservoirs), pump, water bath, water circulator, superfusion block, fraction collector and the collection vials. The temperature probe has been removed.

maintained at 37°C (Grant water circulator, model SU 5). The ten superfusion chambers were housed in a perspex block which was lagged by a water jacket (Fig. 3). A temperature probe was fitted to the block to ensure that the temperature was maintained between 36-37°C (RS, model 612-849). The water circulator maintained the temperature of not only the superfusion block but also the tissue baths, containing buffer. Each chamber had its own stopper to keep its hollow glass cylinder in place and so prevent tissue loss. Tubing was connected to the top of each stopper to enable the effluent to be directed. The effluent of each chamber was collected *via* this outlet tubing being linked to a perspex arm of a modified fraction collector (Gilson micro fraction collector, model 203). This allowed for the effluent of all ten chambers to be collected simultaneously, at 4 minute intervals.

The superfusion system was cleaned at the end of each working day by flushing it through with distilled water for 30 minutes. Also, at the end of each working week the system was flushed with domestic detergent (Decon Laboratories), for 15 minutes followed by distilled water to wash away any detergent.

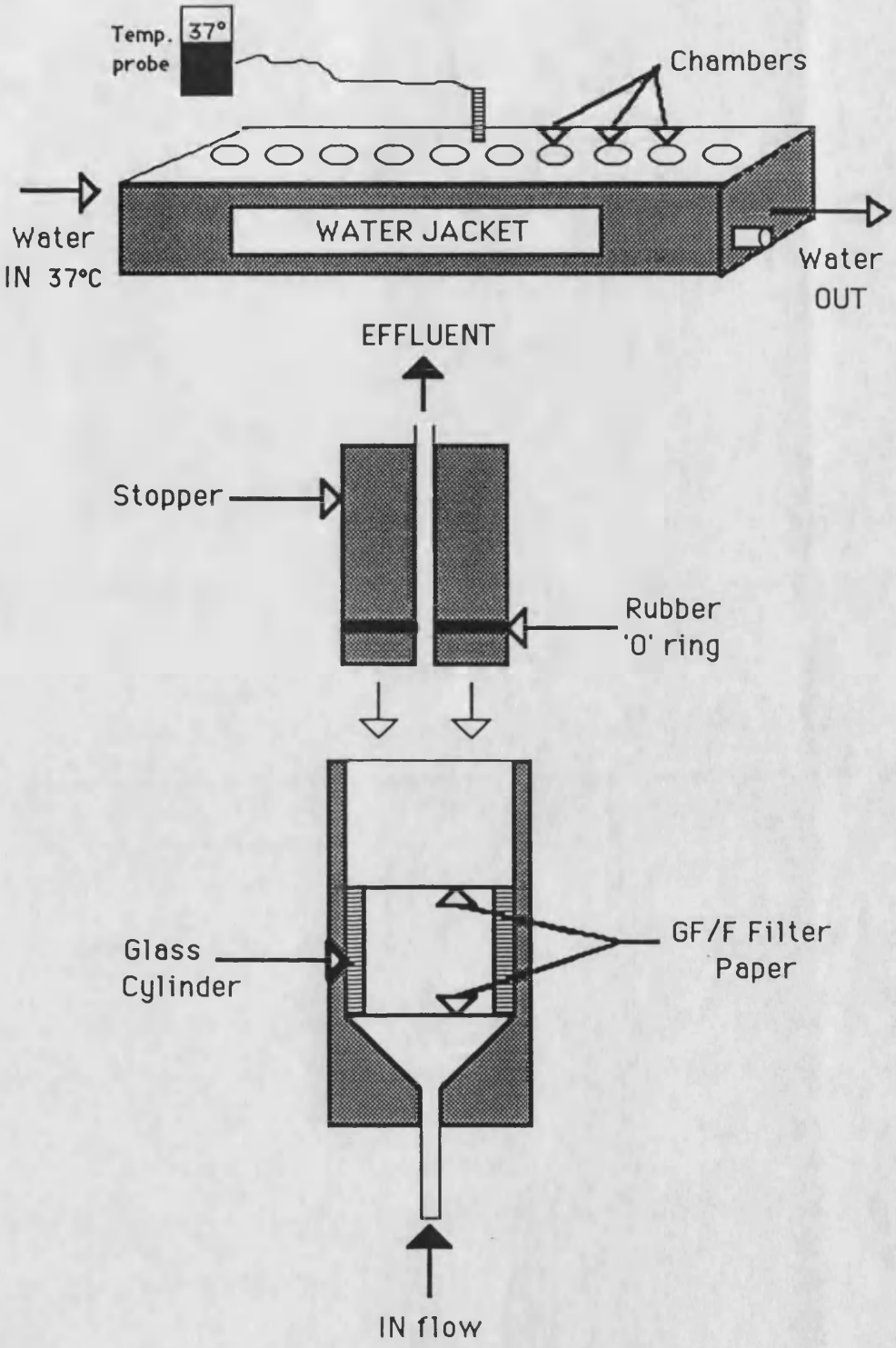
The flow rate for each of the chambers was calibrated at the beginning of each working week. The silicone manifold tubings were regularly inspected and were replaced when any inconsistency in flow rate or the growth of extraneous matter was discovered.

2.3 METHODS

2.3.1 Effect of uptake inhibition on the accumulation of [^{3}H]5-HT into slices of rat cerebral cortex

The cerebral cortex from each of two male Wistar rats (200-350g) was rapidly dissected and chopped in two directions at 250 μm on a McIlwain brain tissue chopper. Half of the slices were incubated for 15 minutes at 37°C (Grant water bath, model JB 2) in 5ml Krebs buffer, p.H. 7.4 (composition (mM) : NaCl 135, KCl 5, $NaHCO_3$ 25, $MgSO_4$ 1, KH_2PO_4 1.25, $CaCl_2$ 2, Glucose 10; gassed with 95% O_2 and 5% CO_2) containing 10 μM pargyline. The other half of the slices were incubated in an identical solution which contained the 5-HT uptake inhibitor, paroxetine (3.2 μM). Following this initial incubation, [^{3}H]5-HT containing ascorbic acid was added to both the tissue slice containing solutions so that the [^{3}H]5-HT and ascorbic acid were present at a concentration of 0.1 μM and 200 μM

Figure 3. THE SUPERFUSION BLOCK



The superfusion block contains ten chambers, an enlargement of one of the chambers is shown above. Tissue slices are placed inside the glass cylinder and the stopper pushed down into place, to prevent their escape.

respectively. Both the solutions were then incubated for a further 15 minutes, at 37°C. At the end of this second incubation period the slices from both solutions were washed three times with 5ml Krebs buffer and 100 µl of slices were transferred to each of four chambers. Each chamber contained a hollow glass cylinder (10 mm high, 8 mm diameter, volume 500 µl) mounted on a piece of circular nylon mesh. After the slices were placed into the glass cylinder, another piece of circular nylon mesh was placed at the top, to prevent the escape of tissue slices. The stoppers were than located into place and the slices superfused against gravity with Krebs buffer (containing 3.2 µM paroxetine to prevent the re-uptake of [^{3}H]5-HT), at a rate of 0.4 ml/min. After a 30 minute period of superfusion the slices were removed from the chambers and weighed. After weighing they were transferred to a plastic scintillation vial and 10 ml of Optiphase "Safe" (LKB) added. They were then sonicated (Dawe, Ultrasonics Ltd.) for 15 minutes. After three hours of stabilization in the scintillation counter (LKB, 1215 Rackbeta) their radioactivity content was determined at an efficiency of 37.5%. Each sample was counted for 5 min. The radioactivity remaining in the tissue slices was expressed as disintegrations per minute, per milligram tissue (dpm/mg).

2.3.2 Effect of change in incubation temperature on the accumulation of [^{3}H]5-HT into slices of rat cerebral cortex

The method of this study was essentially similar to that described in section 2.3.1, the only differences being that the initial incubation step with an uptake inhibitor was omitted, and the second incubation with [^{3}H]5-HT and ascorbic acid was performed at 0°C and 37°C.

2.3.3 Effect of varying concentrations of potassium ions on the fractional release rate

This work was carried out to determine a suitable concentration of potassium for evoking the release of tritium from brain slices. The method being essentially the same as that described in section 2.3.2, up to the point where the chambers were perfused with

Krebs buffer containing paroxetine 3.2 μM for 30 min. Thereafter, the chambers were exposed to Krebs buffer of varying potassium ion concentration (5, 15, 25, 35 and 50 mM), containing paroxetine 3.2 μM. The varying concentrations of potassium ion in Krebs buffer were prepared by an appropriate reduction in the NaCl, thus maintaining osmolarity. Each concentration of potassium was exposed to two chambers. The chambers were superfused with these solutions for 16 min, thereafter the two successive 4 minute fractions were collected. Their fractional release rate was calculated following the determination of the radioactivity still retained by the tissue slices. This was done by removing the slices from the chambers and sonicating them with 10 ml of liquid scintillation fluid followed by counting in a liquid scintillation counter.

2.3.4 Effect of removal of calcium ions on the potassium-evoked release of tritium from brain slices

The method used here was essentially similar to that described in section 2.3.3, up to the point where the chambers were exposed to Krebs buffer containing elevated potassium ions (25 mM for rat and 35 mM for guinea-pig) for 16 minutes. After this period, eleven successive 4-minute fractions were collected (ie t=46 min to t=90 min). In order to prevent the re-uptake of [^{3}H]5-HT into serotoninergic neurones, paroxetine (3.2 μM) was present in the superfusion buffer from t=0 min.

From t=54 min, calcium ions were removed from the Krebs buffer (by iso-osmolar replacement of $CaCl_2$ by $MgCl_2$), supplying four of the chambers (of the remaining six chambers, four received elevated potassium containing calcium while two received unmodified Krebs). As before the radioactivity remaining in the slices and in each fraction was determined by liquid scintillation counting.

In one particular experiment, calcium ions were removed at t=54 min and then subsequently reintroduced at t=78 min.

2.3.5 Effects of agonists and antagonists on the basal release of tritium

The agonists and antagonists that were to be used in the superfusion studies were tested individually at their highest concentration to assess their effects on the basal

release of tritium. The agents (8-OH-DPAT 1 μM, 5-HT 1 μM, noradrenaline 10 μM, methiothepin 1μM and phentolamine 100 nM) were tested on slices of the appropriate brain region, being added to the superfusing Krebs buffer at t=54 min and remained present until t=82 min. Three chambers received one drug, three another and the remaining four chambers served as control.

2.3.6 Rat cortical and hippocampal terminal 5-HT autoreceptor activity over 24-hours

These studies were carried out using the cumulative dose-response technique of Frankhuijzen and Mulder (1982), in its modified version as described by Middlemiss (1984 a,b).

Two rats (weighing 200-350g) were used per experiment, they were killed by cervical dislocation in the appropriate phase of the light : dark cycle. The cerebral cortex or hippocampus was then rapidly dissected, trimmed free of white matter and adhesive blood vessels and chopped in two directions at 250 μm on a McIlwain brain tissue chopper. The resulting slices were incubated for 15 minutes at 37°C in 5 ml Krebs buffer, p.H. 7.4, containing 0.1 μM [^{3}H]5-HT, 200 μM ascorbic acid and 10 μM pargyline. After three washes with 5 ml Krebs buffer, (50 μl cortical or 25 μl hippocampal), aliquots of the slices (about 15-30 mg tissue of cerebral cortex), were transferred to the glass cylinder located in each chamber, of a ten chambered superfusion apparatus and superfused against gravity with Krebs buffer at a rate of 0.4 ml/min. In these experiments a piece of filter paper (Whatman, GF/F, 10 mm diameter), was placed at either end of the glass cylinder to prevent loss of tissue slices, this is different from the nylon mesh used in the uptake study. After a 30 minute period of superfusion, eight of the chambers containing the slices were exposed to Krebs buffer containing elevated potassium ions (25 mM, made by the iso-osmolar replacement of NaCl with KCl), for 16 minutes. After this period, fractions were collected every 4 minutes, 18 in all (ie t=46 min to t=118 min). In order to prevent the re-uptake of [^{3}H]5-HT or endogenous 5-HT into serotoninergic neurones, paroxetine (3.2 μM) was present in the superfusion fluid from t=0 minutes.

At the end of each experiment the tissue slices were removed and placed in plastic scintillation vials. After the addition of 12 ml of liquid scintillation fluid (Optiphase "Safe", LKB), to each vial, it was sonicated (Dawe Ultrasonics Ltd.) for 15 minutes. The ordinary fraction samples (*i.e.* those containing effluent only) were not sonicated but instead were vigorously shaken after the addition of scintillation fluid. All samples were allowed three hours of stabilization in the scintillation counter (LKB, 1215 Rackbeta), prior to counting. Each sample was counted for 3 min. The counter first subtracted a predetermined background value, and then corrected for the quenching of each sample by the use of the ratio procedure, against a stored quench curve. The final counts were therefore expressed as disintegrations per minute.

From t=54 min cumulative dose responses to 5-HT were constructed using four concentrations of agonist (30, 100, 300 and 1000 nM), with a 16 minute time interval between each successive increase in 5-HT concentration (preliminary experiments using 5-HT indicated that the maximum effect was present after 12 minutes). At its highest concentration, 5-HT (1000 nM) did not affect the baseline efflux of tritium, basal efflux was therefore taken as the release in the absence of added agonist. The fractional efflux rates were calculated as described by Frankhuijzen and Mulder (1982). The fractional efflux rate was calculated as the fraction of tritium content of slices at the onset of the respective 4-minute collection period. In each experiment involving an agonist, the fractional efflux rate (mean of 2-4 chambers per treatment), was determined for each 4-minute fraction for both basal and potassium-evoked efflux. The subtraction of basal from potassium-evoked efflux values was taken as control potassium-evoked tritium overflow. Drug effects were expressed as the percentage of the relevant control potassium-evoked tritium overflow, measured 16 min after their addition.

Experiments using the natural agonist, 5-HT were performed at four different, equally spaced time points in the 12 hour light : 12 hour dark cycle of the rat (*i.e.* mid-light, mid-dark, end-light, end-dark).

Experiments using the 5-HT autoreceptor antagonist methiothepin were carried out by the addition of the drug at a concentration of 1 μM to the superfusion fluid (elevated potassium), supplying four of the chambers, at t=30 min. Methiothepin was then present

in the buffer, throughout the superfusion. All chambers received elevated potassium (four were used to assess the effect of the agonist in conjunction with the antagonist, three received only the agonist and the remaining three received no drug). These experiments were performed at the same time points as the agonist studies. Basal values from the agonist data were used in the calculation of the apparent pA_2 values for methiothepin.

2.3.7 Rat hippocampal *alpha*$_2$-adrenoceptor activity over 24-hours

The ability of *alpha*$_2$-adrenoceptors to regulate the release of tritium was examined over 24 hours. The experimental procedure employed was the same as that described above, with the exception that a cumulative dose-response curve was constructed using four concentrations of noradrenaline (0.01, 0.1, 1 and 10 μM), while phentolamine (100 nM) was used to antagonize the effect of noradrenaline.

2.3.8 Effect of 8-OH-DPAT on tritium efflux from cerebral cortex slices

This study was carried out in a similar manner to that described in section 2.3.6. Cerebral cortex slices were prepared from rats during the mid-light point of their light : dark cycle. A cumulative dose-response curve was constructed to 8-OH-DPAT (30, 100, 300 and 1000 nM). All chambers received elevated potassium ions (25 mM), three of these received 5-HT in a cumulative manner and four received 8-OH-DPAT, the remaining three served as control. Basal release rate values from previous experiments were used to determine percentage inhibition.

2.3.9 Guinea-pig 5-HT terminal autoreceptor activity over 24-hours

These studies were carried out in exactly the same manner as the work investigating the release of [^{3}H]5-HT from rat cerebral cortex and hippocampal slices (section 2.3.6), with a few modifications. The potassium ion concentration used to evoke tritium efflux from slices was 35 mM, as opposed to the 25 mM used in rat. Also, 40 μl aliquots of cerebral cortex slices, were used in each chamber. Finally, in antagonist studies methiothepin was used at a concentration of 10 nM.

2.3.10 Effect of chronic methiothepin on autoreceptor sensitivity

In order to assess whether changes in sensitivity of terminal 5-HT autoreceptors could be detected, work was carried out to investigate the sensitivity of presynaptic 5-HT autoreceptors in regulating tritium efflux from cortical slices from control and methiothepin injected rats. Control rats received daily injections of saline for 14 days, while another group of rats received methiothepin (10 mg/kg i.p.), daily for 14 days (Maura and Raiteri, 1984). All rats were injected at the same time of the day (corresponding to the mid-light point in their light : dark cycle), and were killed by cervical dislocation, exactly 3 days after the last injection. The cerebral cortex was then rapidly dissected and used in superfusion studies, as described earlier. Autoreceptor sensitivity was assessed using three concentrations of exogenous 5-HT (30, 100, 300 nM). Eight chambers received elevated potassium (four were exposed to the agonist 5-HT, in the concentrations described above, and four were received no drug). The remaining two chambers received unmodified Krebs and were used to determine basal release rates.

2.3.11 Criteria for the inclusion/exclusion of chambers in the calculation of results

Criteria were set to ensure consistency of results. These were :

(1) Results from experiments would only be considered if more than two chambers of each treatment group had worked (*i.e.* shown no irregular, unexpected release rates);

(2) Chambers would be excluded if their initial starting release rate was ± 25% of the mean of the other chambers (this occurred rather frequently at the start of this work and became a rarity towards the end);

(3) The mean fractional release rate of the surrounding chambers would be taken if for some reason one sample exhibited an anomalous release rate (*i.e.* greater than 20% of previous sample);

(4) The entire data for a chamber would be excluded if it exhibited more than one anomalous release rate;

(5) The entire data for a chamber would be excluded if the release rate during sampling was consistently greater than that at the start of sampling.

2.3.12 Calculations and Statistics

Student's t-test was used for comparing the effect of temperature and that of the 5-HT uptake inhibitor paroxetine on the uptake of [^{3}H]5-HT into slices of rat cerebral cortex.

The apparent IC_{50} (concentration of 5-HT required to inhibit the potassium-evoked release by 50%), was calculated for each experiment. This IC_{50} was converted to the pIC_{50} ($-\log_{10}$ of IC_{50}) for each experiment. Apparent pA_2 ($-\log_{10}$ of the molar concentration of antagonist with which the ratio of equi-effective concentrations of agonist in the presence and absence of antagonist is two), values for methiothepin and phentolamine were calculated for each experiment as described by Schlicker and Gothert (1981).

The one-way analysis of variance test was used for comparison of the effect of effect of potassium ions and its enhancement by methiothepin as well as the apparent pIC_{50} agonist and the apparent pA_2 antagonist values, at the four time points for each brain region. In experiments where autoreceptor sensitivity was determined at two diametrically opposite time points, the Student's t-test was used for comparison.

2.3.13 Drugs

[^{3}H]5-HT, 12-20 Ci/mM (Amersham Int.,Amersham, England), 8-hydroxy-2-(di-n-propylamino)tetralin (Semat Technical Ltd., St. Albans, England), 5-hydroxytryptamine creatnine sulphate (Sigma, St. Louis, MO, USA), noradrenaline bitartrate (Sigma), pargyline hydrochloride (Sigma), paroxetine hydrochloride (gift from Beechams, Brentford, England), phentolamine mesylate ("Rogitine", Ciba, Horsham, England), methiothepin maleate (gift from Roche, Herts, England).

2.4 RESULTS

The selective accumulation of [^{3}H]5-HT into serotoninergic neurones was performed using [^{3}H]5-HT at a concentration of 0.1 μM, a value corresponding to the K_m of the high affinity uptake site (Shaskan and Snyder, 1970 ; Azmitia and Marovitz, 1980).

2.4.1 Effect of uptake inhibition or change in incubation temperature on the accumulation of [^{3}H]5-HT into slices of rat cerebral cortex

Incubations performed at 0°C were associated with a significant, 80%, reduction (37°C 3279±426 dpm/mg tissue, n=9 ; 0°C 664±40, n=4 (mean±s.e.m.)) in the accumulation of [^{3}H]5-HT as compared to those performed at 37°C (Fig. 4).

Paroxetine is a 5-HT uptake inhibitor (Habert *et al.*, 1985). Its inclusion at a concentration of 3.2 μM in the incubation medium produced a significant reduction in the accumulation of [^{3}H]5-HT by slices of rat cerebral cortex (Fig. 4). A reduction of about 84% (control 3279±426 dpm/mg tissue, n=9 ; paroxetine 525±149, n=5 (mean±s.e.m.)) was observed, while the remaining 16% is likely to reflect either non-specific uptake or binding of [^{3}H]5-HT.

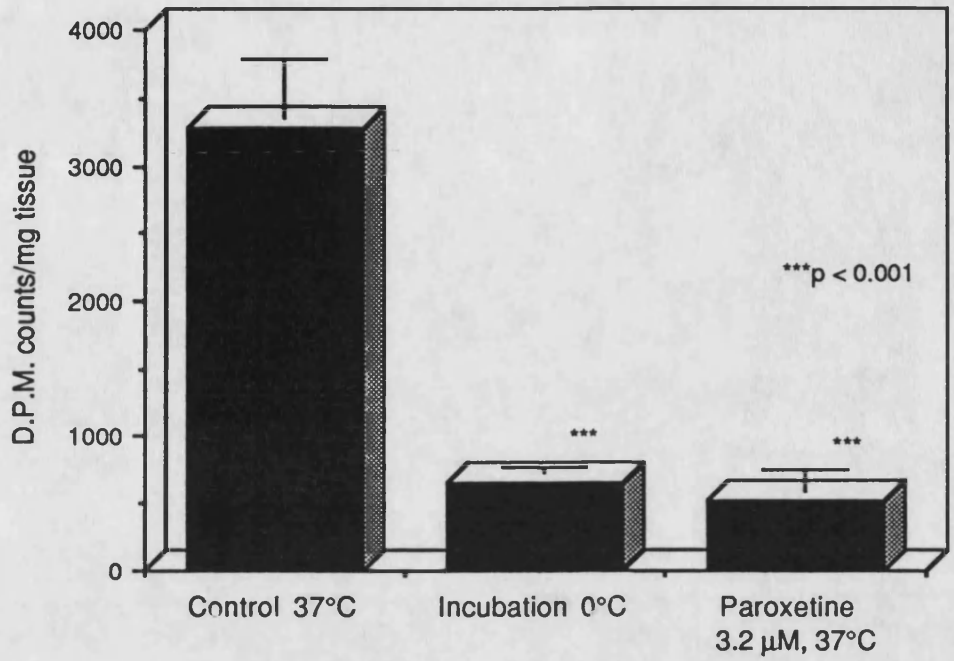
2.4.2 Effect of variation in potassium ion concentration on the fractional release rate from brain slices

The fractional release rate of tritium was dependent on the potassium ion concentration of the Krebs buffer. When potassium ion concentration was plotted against fractional release rate a similar shaped curve was observed from slices of rat cerebral cortex (Fig. 5) and hippocampus (Fig. 6) and guinea-pig cerebral cortex (Fig. 7). Subsequent studies employed a sub-maximal 25 mM potassium ion concentration to evoke the release of tritium from slices of rat cerebral cortex and hippocampus. In contrast, guinea-pig cerebral cortex slices were less sensitive to the effect of potassium ions, consequently a 35 mM concentration was chosen to evoke release. This choice of potassium ion concentration represents a sub-maximal value, since the inhibition of release produced by 5-HT autoreceptor agonists is inversely related to the size of the stimulation (Gothert, 1980).

2.4.3 Effect of the removal of calcium ions on the potassium-evoked release of tritium from brain slices

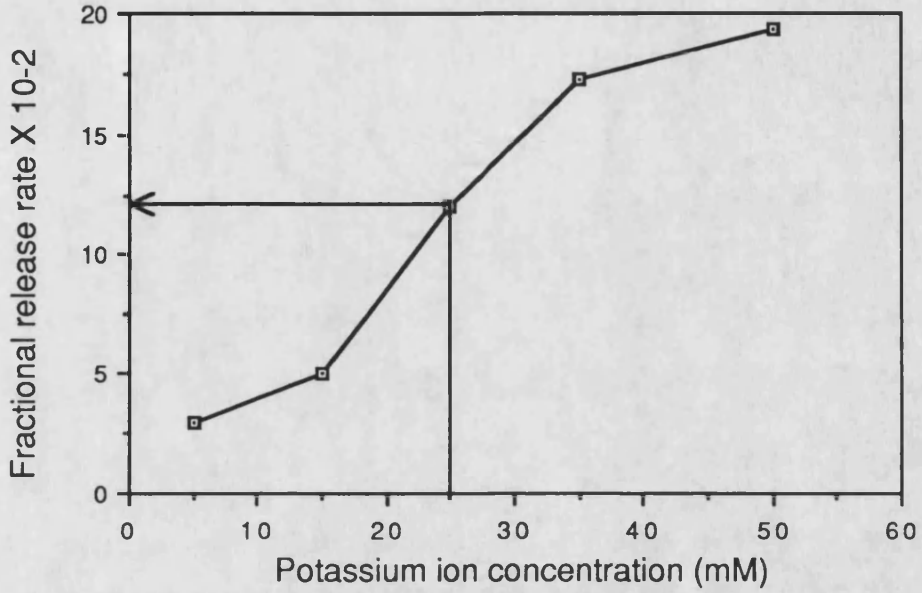
The iso-osmotic replacement of calcium ions in the Krebs buffer with magnesium ions caused the potassium-evoked release of tritium to fall towards basal levels from rat

Figure 4. Effect of a change in incubation temperature or uptake inhibition on the accumulation of [^{3}H]5-HT by rat cerebral cortex slices



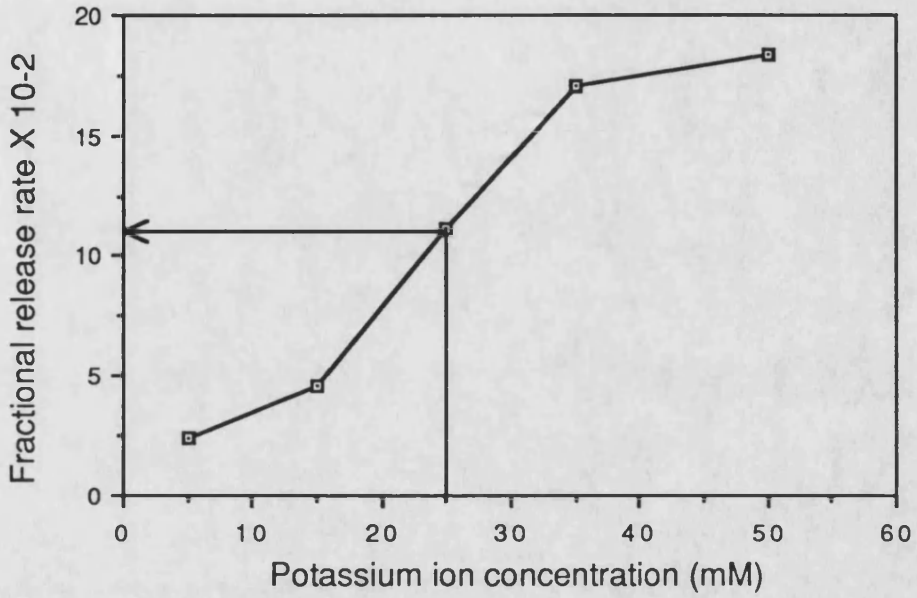
Slices of rat cerebral cortex were incubated with [^{3}H]5-HT for 15 min at 0°C, 37°C and in the presence of the 5-HT uptake inhibitor, paroxetine. The slices were then washed using a superfusion apparatus for 30 min and their radioactivity content determined. The results are expressed as means ± s.e.m. of 4-5 separate experiments.

Figure 5. Effect of varying potassium ion concentration on the release of [^{3}H]5-HT from slices of rat cerebral cortex



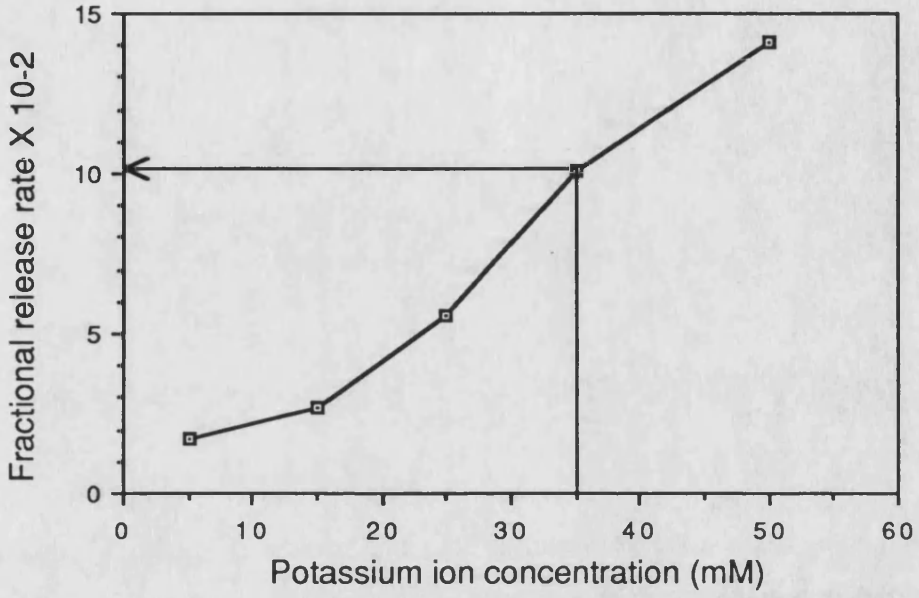
Tissue slices were exposed to a variety of concentrations of potassium ions at t=30 min and the fractional release rate measured at t=46 min. The results are expressed as the mean of the fractional release rate, from two separate experiments.

Figure 6. Effect of varying potassium ion concentration on the release of [^{3}H]5-HT from slices of rat hippocampus



Tissue slices were exposed to a variety of concentrations of potassium ions at t=30 min and the fractional release rate measured at t=46 min. The results are expressed as the mean of the fractional release rate, from two separate experiments.

Figure 7. Effect of varying potassium ion concentration on the release of [^{3}H]5-HT from slices of guinea-pig cerebral cortex



Tissue slices were exposed to a variety of concentrations of potassium ions at t=30 min and the fractional release rate measured at t=46 min. The results are expressed as the mean of the fractional release rate, from two separate experiments.

cerebral cortical (Fig. 8) and hippocampal (Fig. 9) and guinea-pig cerebral cortical (Fig. 10) slices. Interestingly, the reintroduction of calcium ions was associated with an increased release of tritium from rat cerebral cortex slices (Fig. 11). These results suggest that the potassium-evoked release component is entirely calcium dependent, and are in good agreement with the findings of Gothert (1980).

2.4.4 Effect of agonists and antagonists on the basal efflux of tritium

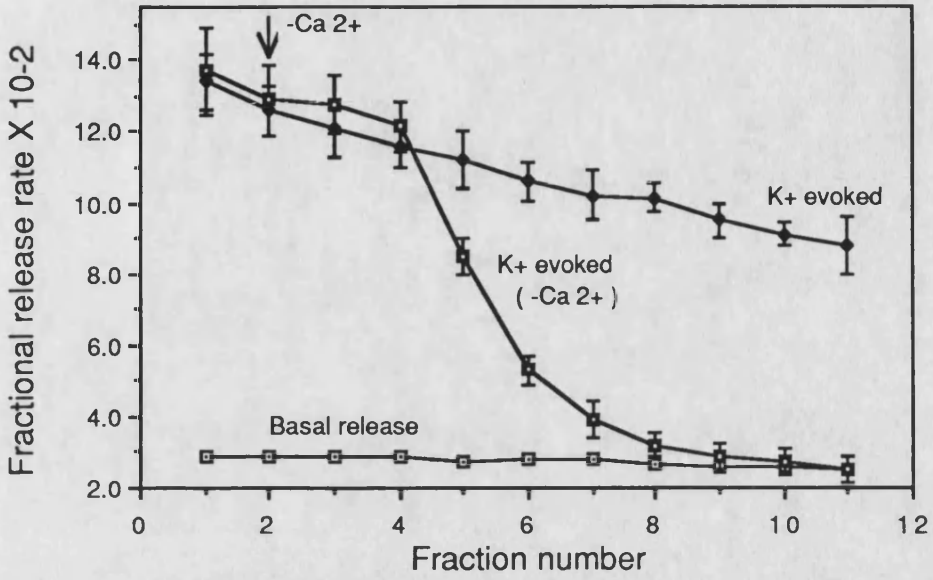
The agonists and antagonists used in superfusion studies were tested individually at their highest concentration for effects on basal tritium efflux. Basal tritium efflux is considered to represent mainly intracellular formed deaminated metabolites (Moret and Briley, 1988). Methiothepin (1 μM), 8-OH-DPAT (1 μM) and 5-HT (1 μM) had no effect on the basal efflux of tritium from rat cerebral cortex slices. In addition, 5-HT (1 μM), noradrenaline (10 μM), methiothepin (1 μM) and phentolamine (100 nM) were without effect on basal tritium efflux from rat hippocampal slices. Furthermore, 5-HT (1 μM) and methiothepin (10 nM) were devoid of any significant effect on the basal efflux of tritium from guinea-pig cerebral cortex slices. The results are shown more concisely in Table 2.

2.4.5 Effect of agonist and antagonist on tritium efflux from rat cerebral cortex slices

The superfusion of [^{3}H]5-HT preloaded rat cerebral cortex slices with continuously elevated (25 mM) potassium ions caused an increased efflux of tritium. This potassium-evoked efflux, measured at the start of the collection period (t=46 min), was virtually identical and not statistically different, at the four time points (Table 3). Baseline efflux at t=46 min was also similar at these time points. At the end of the superfusion period (t=118 min), basal and potassium-evoked efflux rates in the absence of exogenous 5-HT had dropped to about 80% and 40% respectively of their value at the start of the collection period (Table 3).

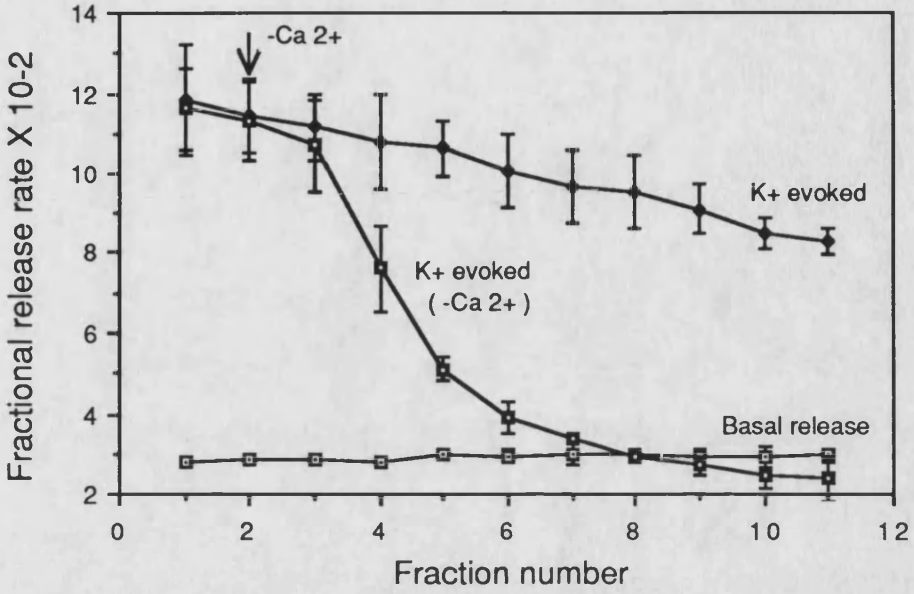
The addition of 5-HT (30 nM to 1 μM) in a cumulative manner to the superfusion buffer caused a dose related inhibition of the potassium-evoked tritium efflux (Fig. 12). The maximum effect, observed at 1 μM 5-HT, represented about 70% inhibition of the

Figure 8. Calcium dependency of the potassium-evoked release of [^{3}H]5-HT from slices of rat cerebral cortex



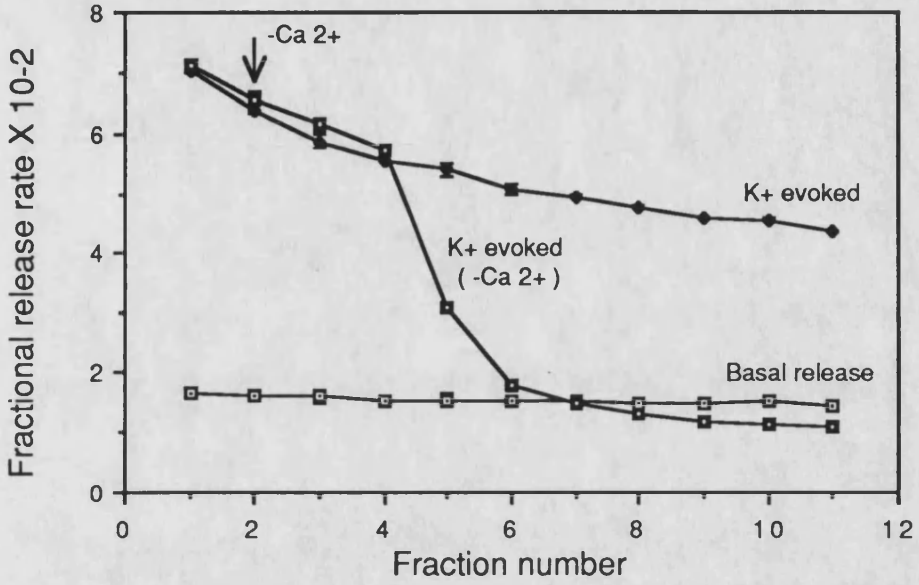
Tissue slices were exposed to Krebs buffer containing elevated potassium ions (25 mM) at t=30 min, to evoke tritium efflux. At t=54 min, calcium ions were removed from the Krebs buffer (by iso-osmolar replacement with magnesium ions), supplying some of the chambers. The results are expressed as the mean ± s.e.m. of the fractional release rate, from two separate experiments.

Figure 9. Calcium dependency of the potassium-evoked release of [^{3}H]5-HT from slices of rat hippocampus



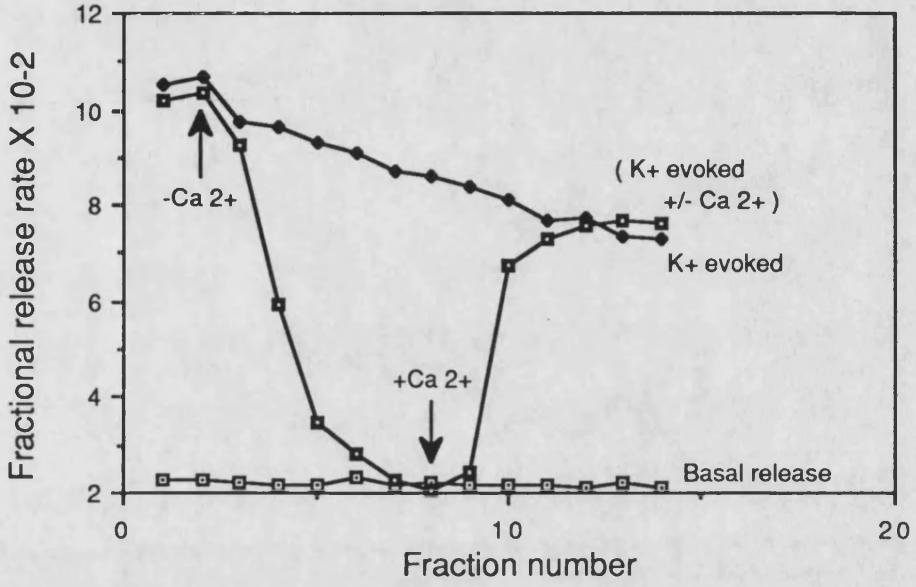
Tissue slices were exposed to Krebs buffer containing elevated potassium ions (25 mM) at t=30 min, to evoke tritium efflux. At t=54 min, calcium ions were removed from the Krebs buffer (by iso-osmolar replacement with magnesium ions), supplying some of the chambers. The results are expressed as the mean ± s.e.m. of the fractional release rate, from two separate experiments.

Figure 10. Calcium dependency of the potassium-evoked release of [^{3}H]5-HT from slices of guinea-pig cerebral cortex



Tissue slices were exposed to Krebs buffer containing elevated potassium ions (35 mM) at t=30 min, to evoke tritium efflux. At t=54 min, calcium ions were removed from the Krebs buffer (by iso-osmolar replacement with magnesium ions), supplying some of the chambers. The results are expressed as the mean ± s.e.m. of the fractional release rate, from two separate experiments.

Figure 11. Effect of calcium replacement (with Mg^{2+}) and its subsequent reintroduction on the potassium-evoked release of [^{3}H]5-HT from slices of rat cerebral cortex



Tissue slices were exposed to Krebs buffer containing elevated potassium ions (25 mM) at t=30 min, to evoke tritium efflux. At t=54 min, calcium ions were removed from the Krebs buffer (by iso-osmolar replacement with magnesium ions), supplying some of the chambers and reintroduced at t=78 min. The results are expressed as the mean of the fractional release rate, from a single experiment.

Table 2. Effect of 5-HT receptor agonists and antagonists on the basal release of tritium from brain slices

Species	Drug	Release rate (% tissue stores/4 min)
Rat cerebral cortex		
	Control	2.947±0.082
	5-HT (1 μM)	2.762±0.072
	Methiothepin (1 μM)	3.089±0.068
	Control	2.851±0.071
	8-OH-DPAT (1 μM)	2.713±0.090
Rat hippocampus	Control	2.480±0.075
	5-HT (1 μM)	2.245±0.075
	Methiothepin (1 μM)	2.594±0.084
	Control	2.565±0.052
	Noradrenaline (10 μM)	2.402±0.085
	Phentolamine (100 nM)	2.461±0.064
Guinea pig cerebral cortex	Control	1.681±0.043
	5-HT (1 μM)	1.645±0.067
	Methiothepin (10 nM)	1.733±0.064

The results are expressed as the means ± s.e.m. of 2-4 separate experiments. The value for each experiment is itself the mean of 2-4 measurements. The drugs were added to the superfusion buffer at t=54 min and their effect on basal release determined at t=82 min. The superfusion buffer contained 3.2 μM paroxetine, throughout the experiment to prevent the re-uptake of 5-HT. The data was analysed using Student's t-test.

Table 3. Release parameters for tritium from slices of rat cerebral cortex, measured at various time points of the light : dark cycle

---

Hours after lights on = **0 (End-dark)**

basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
3.154±0.208	2.779±0.192

potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
9.960±0.559	4.043±0.286

maximum inhibition : 1μM 5-HT = 72.00±4.15% (n=5)

potassium-evoked release above basal (t=46 min, n=5) = 11.516±0.179

methiothepin enhancement (t=46 min, n=5) = 13.226±0.201***

Hours after lights on = **6 (Mid-light)**

basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
3.142±0.186	2.805±0.188

potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
10.360±0.172	3.588±0.322

maximum inhibition : 1μM 5-HT = 76.42±3.37% (n=5)

potassium-evoked release above basal (t=46 min, n=5) = 12.068±0.295

methiothepin enhancement (t=46 min, n=5) = 13.873±0.340**

Hours after lights on = **12 (End-light)**

basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
3.278±0.178	2.869±0.179

potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
10.395±0.568	3.721±0.416

(continued on following page)

Table 3. cont.

maximum inhibition : 1μM 5-HT = 78.193±6.80% (n=5)

potassium-evoked release above basal (t=46 min, n=5) = 12.005±0.215
methiothepin enhancement (t=46 min, n=5) = 13.799±0.125***

Hours after lights on = **18 (Mid-dark)**
basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
3.160±0.082	2.535±0.103

potassium-evoked release above basal (% tissue stores/4 min, n=5)

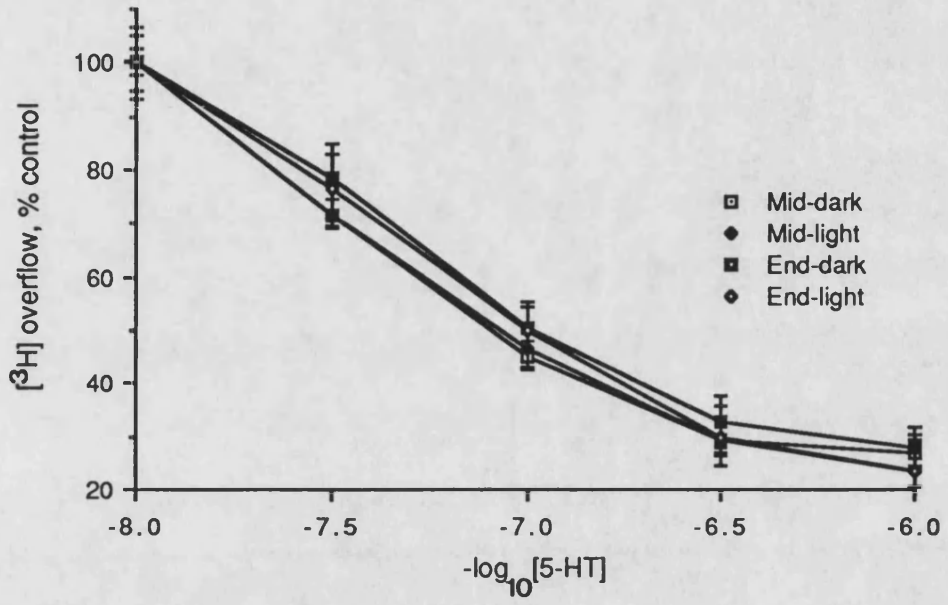
t=46 min	t=118 min
10.06±0.367	4.197±0.107

maximum inhibition : 1μM 5-HT = 72.93±2.52% (n=5)

potassium-evoked release above basal (t=46 min, n=4) = 11.773±0.460
methiothepin enhancement (t=46 min, n=4) = 13.953±0.300**

The results are expressed as means ± s.e.m. of the number of separate experiments in parenthesis. The value for each experiment is itself the mean of 2-4 measurements. The potassium (25mM) evoked release rate above basal was obtained by subtracting the basal release rate from the potassium-evoked component, in the absence of exogenous 5-HT. The percentage inhibition is calculated assuming a maximum effect at 1μM exogenous 5-HT. Methiothepin (1μM) was added at t=30 min and its effect on the potassium (25mM) evoked release was measured at t=46. Student's t-test was used for assessing the enhancing effect of methiothepin on the potassium-evoked release of tritium, **$p<0.01$; ***$p<0.001$. The one-way analysis of variance test was used to compare the effect of potassium ions and methiothepin, at the four time points.

Figure 12. Dose response relationship of the inhibitory effect of 5-HT on [^{3}H]5-HT efflux from slices of rat cerebral cortex induced by continuous potassium (25 mM) stimulation



The results are the means ± s.e.m. of 5 separate experiments for each time point.

potassium-evoked efflux at the four time points tested (Table 3). This maximum inhibition was used to calculate the apparent pIC_{50} value for each experiment, at the four time points of the light : dark cycle (Fig. 13). Statistical analysis did not reveal any significant differences. Interestingly, while the addition of exogenous 5-HT (100 nM) attenuated the potassium-evoked efflux of tritium, its removal produced complete recovery (Fig. 14).

Methiothepin (1 μM) when added to the superfusion buffer at t=30 min, caused a significant enhancement of the potassium-evoked efflux of tritium at t=46 min, at all four time points tested (Table 3). Statistical analysis, was unable to expose any significant differences in the level of enhancement produced by methiothepin at these time points. This concentration of methiothepin also attenuated the effect of 5-HT (30 nM to 1 μM) added in a cumulative manner at these time points. The apparent pA_2 values calculated were virtually identical (Fig. 15) and not statistical different.

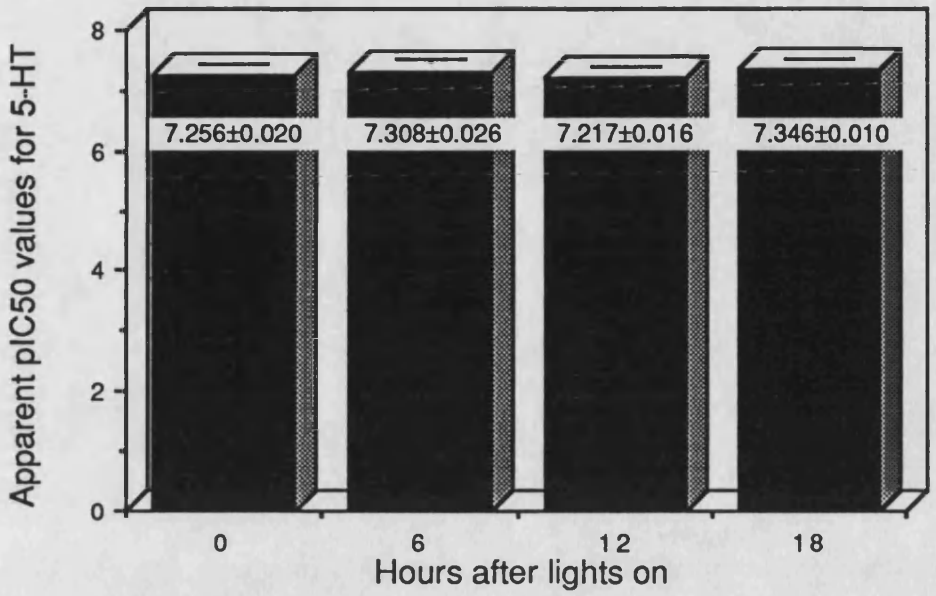
The apparent pIC_{50} value for 5-HT and the apparent pA_2 value for methiothepin calculated in the present study, are identical to those reported by Middlemiss (1984a,b), when measuring the release of 5-HT from the rat frontal cortex.

2.4.6 Effect of 8-OH-DPAT on tritium efflux from cerebral cortex slices

The cumulative addition of 8-OH-DPAT (30 nM to 1 μM) to the superfusion buffer containing paroxetine 3.2 μM, had little effect on the potassium (25 mM) evoked efflux of tritium (Fig. 16). In contrast, the cumulative addition of 5-HT produced dose related attenuation of the potassium-evoked efflux of tritium. The maximum inhibition observed at 1 μM concentration of 5-HT and 8-OH-DPAT was about 85% and 16% respectively.

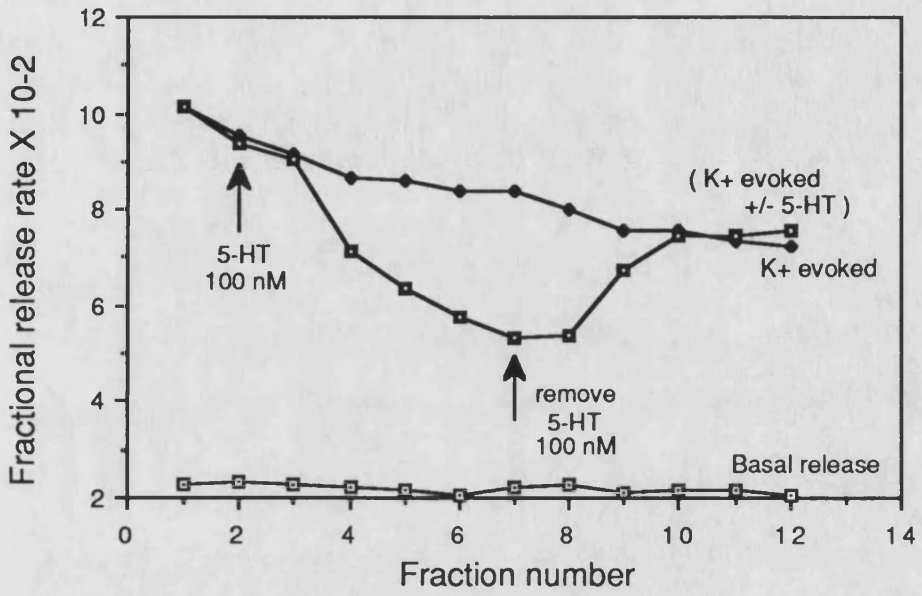
A more extensive study of the effect of 8-OH-DPAT on the terminal 5-HT autoreceptor was conducted by Middlemiss (1984b). In general, 8-OH-DPAT was reported to be devoid of any agonist or antagonist activity when tested up to a concentration of 1 μM.

Figure 13. Apparent pIC_{50} values for the inhibition of the release of [^{3}H]5-HT from slices of rat cerebral cortex by 5-HT



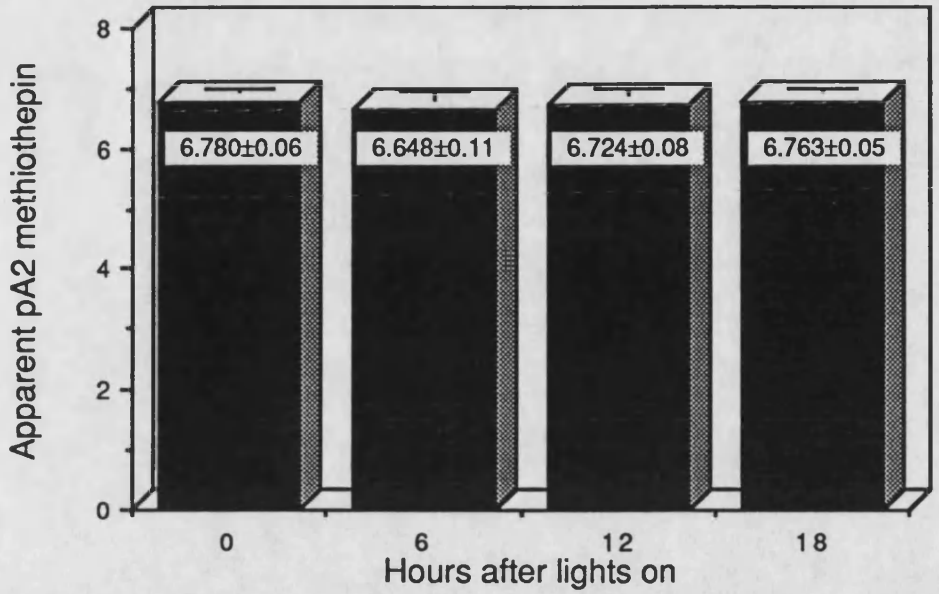
The results are the means ± s.e.m. of 5 separate experiments for each time point.

Figure 14. Effect of 5-HT (100 nM) and its subsequent removal on the potassium-evoked release of [^{3}H]5-HT from slices of rat cerebral cortex



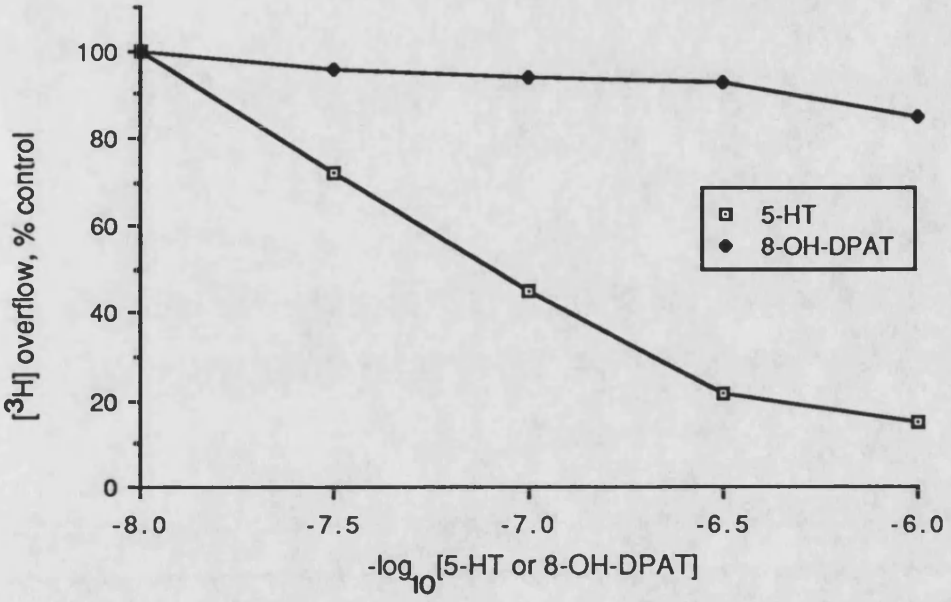
Slices of rat cerebral cortex were exposed to Krebs buffer containing 5-HT (100 nM) at t=54 min and removed at t=74 min. The results are expressed as the mean fractional release rate from a single experiment.

Figure 15. Apparent pA_2 values for methiothepin antagonism of serotonin in slices of rat cerebral cortex



The results are expressed as means ± s.e.m. of 4-5 separate experiments at each time point.

Figure 16. Dose response relationship of the inhibitory effect of serotonin or 8-OH-DPAT on the potassium-evoked release of [^{3}H]5-HT from slices of rat cerebral cortex, determined at mid-light



The values represent the means of a single experiment.

2.4.7 Effect of agonist and antagonist on tritium efflux from rat hippocampal slices

Continuously elevated (25 mM) potassium ion concentration in the superfusion buffer increased tritium efflux from rat hippocampal slices. The potassium-evoked efflux measured at t=46 min was much lower than that observed from rat cerebral cortex slices but was similar at all four time points (Table 4). Statistical analysis, showed that the potassium-evoked efflux was not significantly different at these time points. Baseline efflux at t=46 min was also very similar, except for the values obtained at mid-dark [2.420±0.194% tissue stores/4 min (mean ± s.e.m., n=5)] were much lower. At the end of the superfusion period (t=118 min), basal and the potassium-evoked efflux rates in the absence of exogenous 5-HT decreased to about 94% and 36% respectively of their value at the beginning of the collection period (Table 4).

When added in a cumulative manner to the superfusion buffer 5-HT (30 nM to 1 μM) caused a dose related inhibition in the potassium-evoked efflux of tritium (Fig. 17). The maximum effect, observed at 1 μM 5-HT was comparable to that observed for cerebral cortex slices. The inhibition produced at end-dark (78%) and at end-light (81%) was greater as compared to mid-dark (62%) or mid-light (67%), see Table 4. The apparent pIC_{50} values calculated using these inhibitions were not statistically different at the four time points (Fig. 18).

The addition of methiothepin (1 μM) to the superfusion buffer at t=30 min produced a significant enhancement of the potassium-evoked efflux of tritium at all four time points tested, measured at t=46 min (Table 4). The magnitude of this enhancement was not significantly different at the four time points. The presence of methiothepin in the superfusion buffer attenuated the effect of 5-HT (30 nM to 1 μM) added cumulatively at all four time points. The apparent pA_2 values calculated for this antagonism were not statistically different at these time points (Fig. 19).

When added in a cumulative manner to the superfusion buffer, noradrenaline in the concentration range 10 nM to 10 μM caused a dose related inhibition of the potassium-evoked efflux of tritium, at all four time points tested (fig. 20). The maximum effect, observed at 10 μM noradrenaline was similar at these time points and was of the order

Table 4. Release parameters for tritium from slices of rat hippocampus, measured at various time points of the light : dark cycle

---

Hours after lights on = **0 (End-dark)**

basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
3.021±0.137	2.856±0.185

potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
9.865±0.392	3.578±0.333

maximum inhibition : 1μM 5-HT = 78.12±6.15% (n=5)
10μM noradrenaline = 82.55±2.69% (n=5)

potassium-evoked release above basal (t=46 min, n=5) = 9.319±0.215
methiothepin enhancement (t=46 min, n=5) = 11.206±0.270***

Hours after lights on = **6 (Mid-light)**

basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
2.828±0.076	2.591±0.140

potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
9.156±0.562	3.393±0.198

maximum inhibition : 1μM 5-HT = 66.96±4.71% (n=5)
10μM noradrenaline = 78.69±5.26% (n=5)

potassium-evoked release above basal (t=46 min, n=5) = 9.634±0.640
methiothepin enhancement (t=46 min, n=5) = 11.987±0.468*

potassium-evoked release above basal (t=46 min, n=4) = 7.438±0.269
phentolamine (t=46 min, n=4) = 8.009±0.162

Hours after lights on = **12 (End-light)**

Basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
3.004±0.172	2.892±0.139

potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
9.078±0.276	3.395±0.173

(continued on following page)

Table 4. cont.

maximum inhibition : 1μM 5-HT = 81.33±3.53% (n=5)
10μM noradrenaline = 78.15±3.31% (n=6)

potassium-evoked release above basal (t=46 min, n=5) = 9.129±0.151
methiothepin enhancement (t=46 min, n=5) = 11.501±0.206***

Hours after lights on = **18 (Mid-dark)**
basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
2.420±0.194	2.332±0.245

potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
9.717±0.123	3.597±0.307

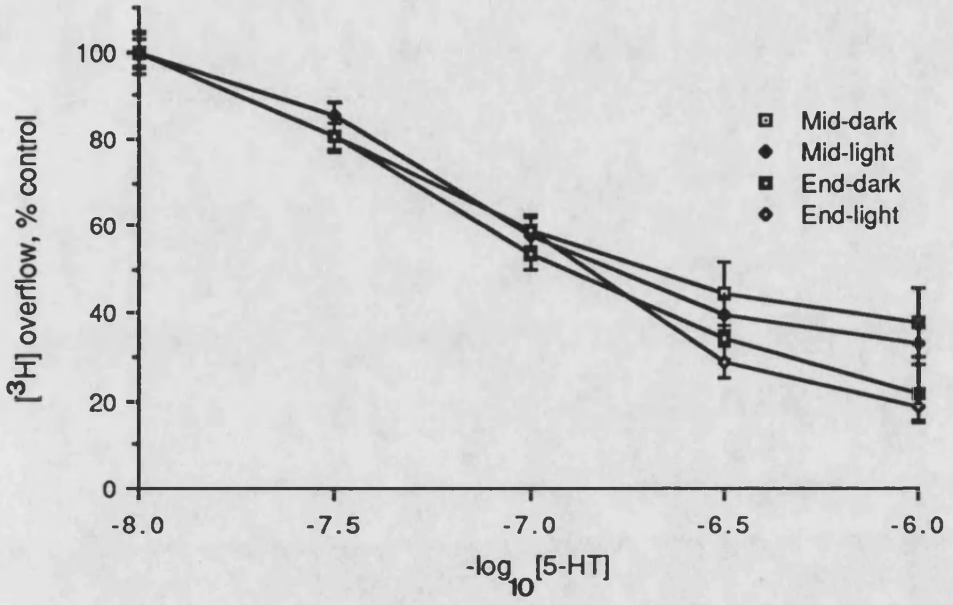
maximum inhibition : 1μM 5-HT = 62.38±7.78% (n=5)
10μM noradrenaline = 70.04±5.85% (n=5)

potassium-evoked release above basal (t=46 min, n=5) = 9.953±0.242
methiothepin enhancement (t=46 min, n=5) = 11.908±0.216***

potassium-evoked release above basal (t=46 min, n=5) = 7.947±0.100
phentolamine (t=46 min, n=5) = 8.356±0.092*

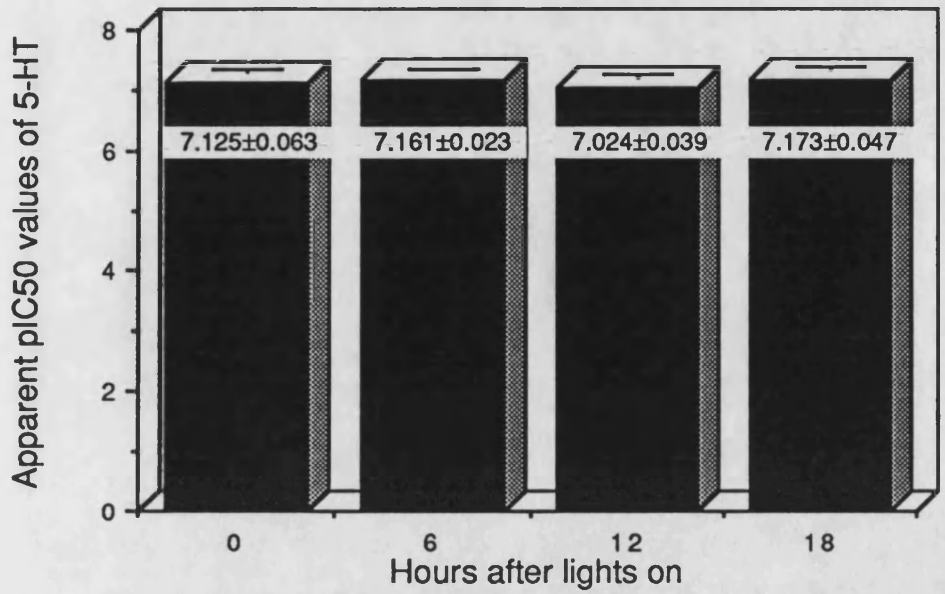
The results are expressed as means ± s.e.m. of the number of separate experiments in parenthesis. The value for each experiment is itself the mean of 2-4 measurements. The potassium (25mM) evoked release rate above basal was obtained by subtracting the basal release rate from the potassium-evoked component, in the absence of exogenous 5-HT. The percentage inhibition is calculated assuming a maximum effect at either 1μM exogenous 5-HT or 10μM noradrenaline. Methiothepin (1μM) or phentolamine (100nM) was added at t=30 min and its effect on the potassium-evoked release of tritium was measured at t=46. Student's t-test was used for comparison of the potassium-evoked release with the methiothepin or phentolamine added group, *$p<0.05$; ***$p<0.001$. Furthermore, this statistical test was also used for the analysis of the effect of phentolamine on the potassium-evoked release of tritium. In contrast, the one-way analysis of variance test was used to compare the effects of potassium ions and methiothepin, at the four time points.

Figure 17. Dose response relationship of the inhibitory effect of 5-HT on [^{3}H]5-HT efflux from slices of rat hippocampus induced by continuous potassium (25 mM) stimulation



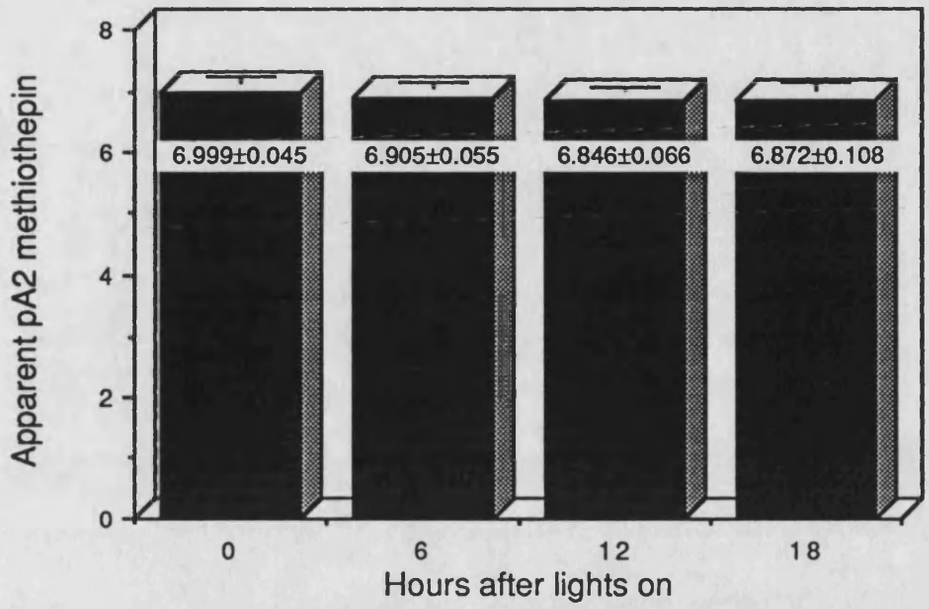
The results are the means ± s.e.m. of 5 separate experiments at each time point.

Figure 18. Apparent pIC_{50} values for the inhibition of the release of [^{3}H]5-HT from slices of rat hippocampus by 5-HT



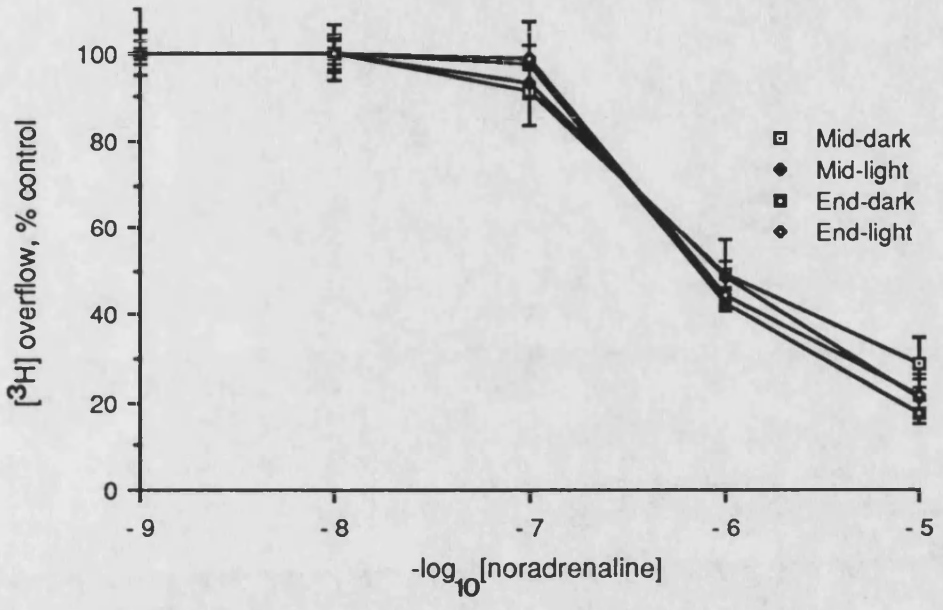
The results are the means ± s.e.m. of 5 separate experiments at each time point.

Figure 19. **Apparent pA_2 values for methiothepin antagonism of serotonin in slices of rat hippocampus**



The results are expressed as means ± s.e.m. of 5 separate experiments for each time point.

Figure 20. Dose response relationship of the inhibitory effect of noradrenaline on [^{3}H]5-HT efflux from slices of rat hippocampus induced by continuous potassium (25 mM) stimulation



The results are the means ± s.e.m. of 5-6 experiments at each time point.

seen using 1 μM 5-HT (Table 4). The apparent pIC_{50} values were also very similar at the four time points (Fig. 21). Statistical analysis was unable to reveal any significant differences in these values.

Phentolamine (100 nM) when added to the superfusion buffer caused a significant enhancement of the potassium-evoked efflux of tritium at mid-dark but not at mid-light (Table 4). its presence in the superfusion buffer caused the dose-response curve for noradrenaline to be shifted to the right, both, at mid-dark and at mid-light. The apparent pA_2 values calculated at these time points were not statistically different (Fig. 22).

The apparent pIC_{50} and apparent pA_2 values agree well with those reported by Gothert *et al.* (1981) for the release of 5-HT from cerebral cortex slices.

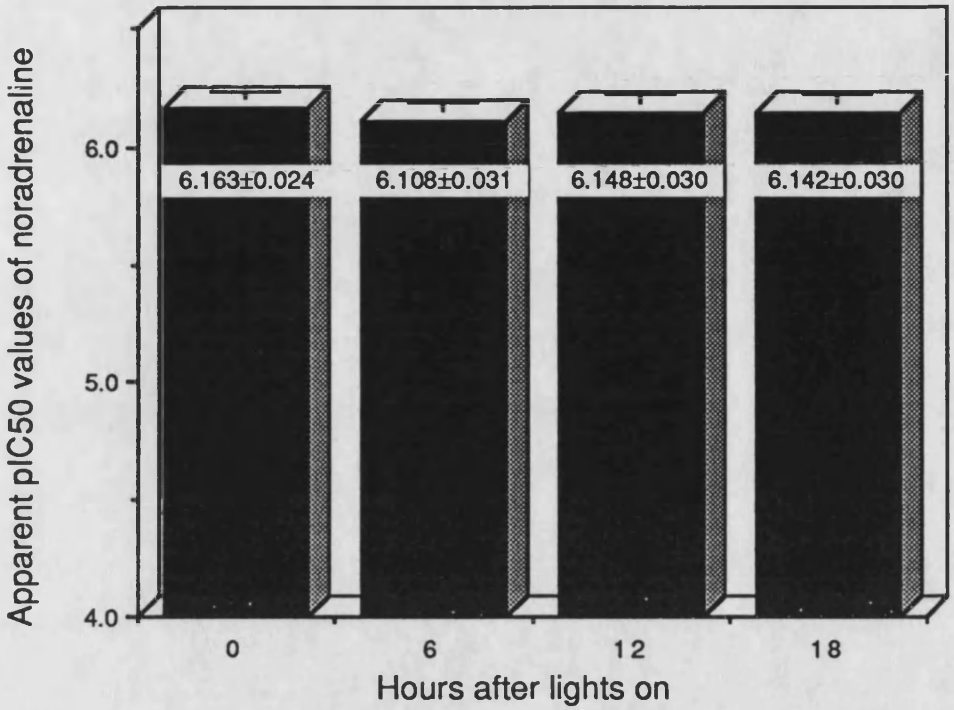
2.4.8 Effect of agonist and antagonist on tritium efflux from guinea-pig cerebral cortex slices

Superfusion of guinea-pig cerebral cortex slices preloaded with [^{3}H]5-HT with continuously elevated (35 mM) potassium ions resulted in an increased efflux of tritium. This effect of potassium, measured at the start of the collection period (t=46 min) was lower than that measured in the rat cerebral cortex or hippocampus, but was essentially similar in magnitude at the four time points sampled (Table 5), and not statistically different. At the end of the superfusion period (t=118 min), basal and potassium-evoked efflux rates had declined to about 80% and 40% respectively of their value at the beginning of the collection period (Table 5).

The cumulative addition of 5-HT (30 nM to 1 μM) to the superfusion buffer caused a dose-related inhibition of the potassium-evoked efflux of tritium at all four time points (Fig. 23). The maximum inhibition occurred at about 1 μM 5-HT and represented a reduction of about 68% of the potassium-evoked efflux at each of the four time points (Table 5). The apparent pIC_{50} values were calculated at each of these time points assuming a maximum effect at 1 μM 5-HT (Fig. 24). Statistical analysis did not reveal any significant differences.

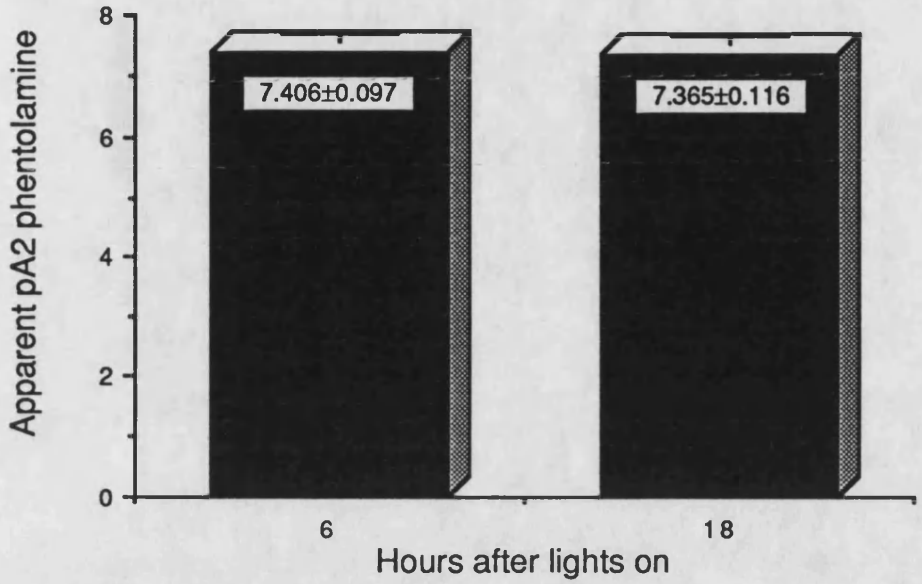
Methiothepin (10 nM) added to the superfusion buffer at t=30 min caused a

Figure 21. Apparent pIC_{50} values for the inhibition of the release of [^{3}H]5-HT from slices of rat hippocampus by noradrenaline



The results are the means ± s.e.m. of 5-6 experiments at each time point.

Figure 22. Apparent pA_2 values for phentolamine antagonism of noradrenaline in slices of rat hippocampus, determined at mid-light and mid-dark



The results are expressed as means ± s.e.m. of 4-5 experiments for each time point.

Table 5. Release parameters for tritium from slices of guinea-pig cerebral cortex, measured at various time points of the light : dark cycle

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Hours after lights on = **0 (End-dark)**

basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
1.706±0.042	1.370±0.024

potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
6.896±0.164	2.919±0.058

maximum inhibition : 1µM 5-HT = 68.41±2.06% (n=5)

Hours after lights on = **6 (Mid-light)**

basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
1.759±0.055	1.423±0.040

potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
7.243±0.168	2.947±0.098

maximum inhibition : 1µM 5-HT = 68.64±1.49% (n=5)

potassium-evoked release above basal (t=46 min, n=4) = 6.668±0.082

methiothepin enhancement (t=46 min, n=4) = 7.612±0.049***

Hours after lights on = **12 (End-light)**

basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
1.640±0.032	1.318±0.045

potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
6.764±0.149	2.651±0.111

maximum inhibition : 1µM 5-HT = 67.07±1.24% (n=5)

(continued on following page)

Table 5. cont.

Hours after lights on = **18 (Mid-dark)**

basal release (% tissue stores/4 min, n=5)

t=46 min	t=118 min
1.708±0.041	1.379±0.057

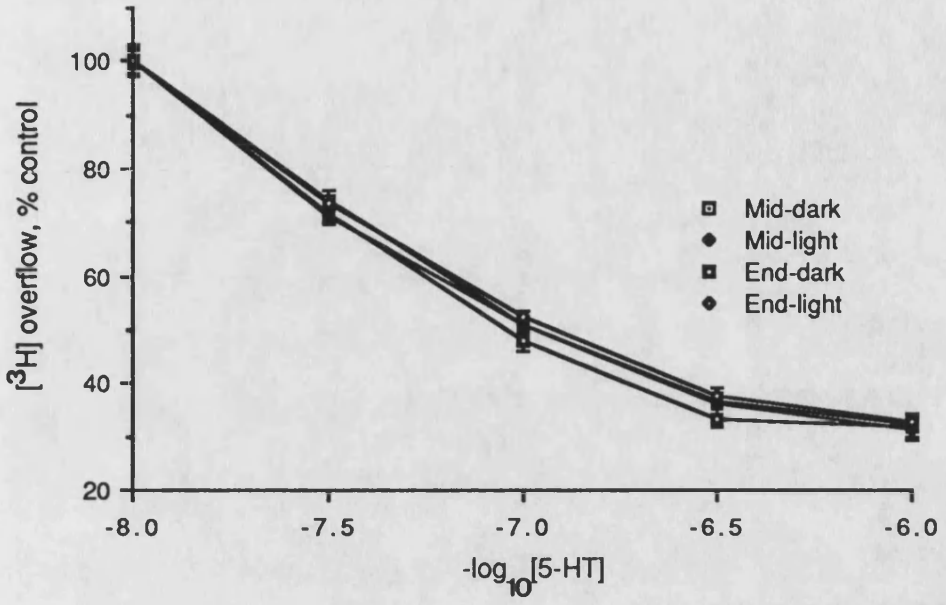
potassium-evoked release above basal (% tissue stores/4 min, n=5)

t=46 min	t=118 min
7.088±0.178	2.878±0.110

maximum inhibition : 1μM 5-HT = 67.59±0.80% (n=5)

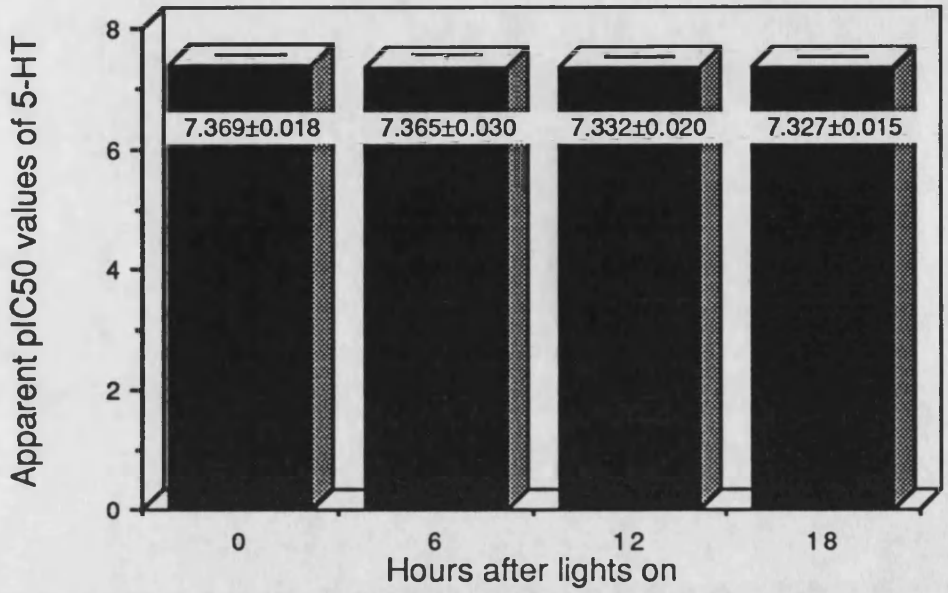
The results are expressed as means ± s.e.m. of the number of separate experiments in parenthesis. Furthermore, the value of each experiment is composed of the mean of 2-4 measurements. The potassium (35mM) evoked release rate above basal was obtained by subtracting the basal release rate from the potassium-evoked component, in the absence of exogenous 5-HT. The percentage inhibition is calculated assuming a maximum effect at 1μM exogenous 5-HT. Methiothepin (10nM) was added at t=30 min and its effect on the potassium-evoked release was measured at t=46. The one-way analysis of variance test was used to compare the effect of potassium at the four time points. Student's t-test was used for comparison of the potassium-evoked release with the methiothepin added group, $^{***}p<0.001$.

Figure 23. Dose response relationship of the inhibitory effect of 5-HT on [^{3}H]5-HT efflux from slices of guinea-pig cerebral cortex induced by continuous potassium (35 mM) stimulation



The results are the means ± s.e.m. of 5 separate experiments for each time point.

Figure 24. Apparent pIC_{50} values for inhibition of the release of [^{3}H]5-HT from slices of guinea-pig cerebral cortex by 5-HT



The results are the means ± s.e.m. of 5 separate experiments at each time point.

significant enhancement of the potassium-evoked efflux of tritium (from slices prepared at mid-light), when measured at t=46 min (Table 5). This concentration of methiothepin attenuated the inhibitory effect of 5-HT (30 nM to 1 μM) when added in a cumulative manner, yielding an apparent pA_2 value of 8.400±0.090 (mean ± s.e.m., n=4).

The apparent pIC_{50} values for 5-HT and the apparent pA_2 value for methiothepin are identical to those obtained by Middlemiss *et al.*, (1988), when measuring the release of 5-HT from the guinea-pig frontal cortex.

2.4.9 Effect of chronic methiothepin on autoreceptor sensitivity

Rats received daily injections for 14 days of either saline or methiothepin (10mg/kg i.p.) at a time point corresponding to mid-light of their light : dark cycle. Saline-injected rats gained weight and looked healthy. In contrast, rats injected with methiothepin were sedated shortly afterwards and lost weight during the first few days but later gained it although at a slower rate compared to saline-injected rats. Water consumption was also reduced in comparison to saline-injected rats. Immediately prior to being killed, 3 days after the last injection, methiothepin-injected rats were about 30% underweight in comparison to saline-injected rats (methiothepin=169±9.118 ; control=243±6.984, mean ± s.e.m., n=6).

Exposure of [^{3}H]5-HT-preloaded cerebral cortex slices to elevated (25 mM) potassium ions produced an increased tritium efflux. This effect of potassium, measured at the start of the collection period (t=46 min) was significantly lower (Table 6) as compared to the mid-light point obtained from previous studies (Table 3). However, the stimulant effect of potassium from slices of methiothepin- and saline-injected rats was similar (Table 6), making it likely that methiothepin had been cleared by the rats. Baseline efflux at t=46 min was not significantly different (Table 6) from that obtained at the mid-light point of previous studies (*cf.* Table 3), and was similar in the injected rats (Table 6). At the end of the superfusion period (t=118 min), basal and potassium-evoked efflux had declined to about 87% and 53% respectively of their value at the start of the collection period (Table 6).

The cumulative addition of 5-HT (30 nM to 300 nM) to the superfusion buffer

Table 6. Release parameters for tritium from cerebral cortex slices of chronically injected rats

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Saline injected daily for 14 days

basal release (% tissue stores/4 min, n=3)

t=46 min	t=102 min
2.563±0.087	2.237±0.100

potassium-evoked release above basal (% tissue stores/4 min, n=3)

t=46 min	t=102 min
7.485±0.042**	3.978±0.064

maximum inhibition : 300 nM 5-HT = 72.42±3.47% (n=3)

Methiothepin (10mg/kg i.p.) injected daily for 14 days

basal release (% tissue stores/4 min, n=3)

t=46 min	t=102 min
2.543±0.103	2.242±0.073

potassium-evoked release, above basal (% tissue stores/4 min, n=3)

t=46 min	t=102 min
7.526±0.053**	3.966±0.113

maximum inhibition : 300 nM 5-HT = 71.38±0.63% (n=3)

Rats were injected daily with either saline or methiothepin (10mg/kg i.p.) for 14 days and killed 3 days after the last injection, at the time point corresponding to the mid-light position of their light : dark cycle. The results are expressed as means ± s.e.m. of the number of separate experiments in parenthesis. The value for each experiment is itself the mean of 2-4 measurements. The potassium (25mM) evoked release rate, above basal was obtained by subtracting the basal release rate from the potassium-evoked component, in the absence of exogenous 5-HT. The percentage inhibition is calculated assuming a maximum effect at 300 nM exogenous 5-HT. Student's t-test was used for comparing the potassium-evoked release from saline- and methiothepin-injected rats with those of non-injected rats, at mid-light, $^{**}p<0.01$.

caused a dose-related inhibition of the potassium-evoked tritium efflux from slices of saline- and methiothepin-injected rats. The inhibition produced by each of the doses of 5-HT was very similar in the saline-injected rats to those previously observed at this time point in non-injected rats (*cf.* Figure 12). In contrast, a greater inhibition was observed in slices from methiothepin-injected rats (30 nM 5-HT : saline=29.92±2.21%, methiothepin=35.90±4.23% ; 100 nM 5-HT : saline=57.87±3.98%, methiothepin=63.77±4.67%, mean ± s.e.m., n=3). However, the inhibition produced by 300 nM 5-HT was very similar in saline- and methiothepin-injected rats. Although in this study a maximum inhibition was assumed to be produced by 300 nM 5-HT (Table 6), it was very similar to that previously observed with 1 μM 5-HT in non-injected rats (*cf.* Table 3). Statistical analysis (Student's t-test) of the apparent pIC_{50} values calculated for the slices from saline- and methiothepin-injected rats shows a significant difference (Fig. 25). In addition, the apparent pIC_{50} value for the saline-injected rats (Table 6) was not significantly different to that previously obtained from non-injected rats at this time point (*cf.* Table 3).

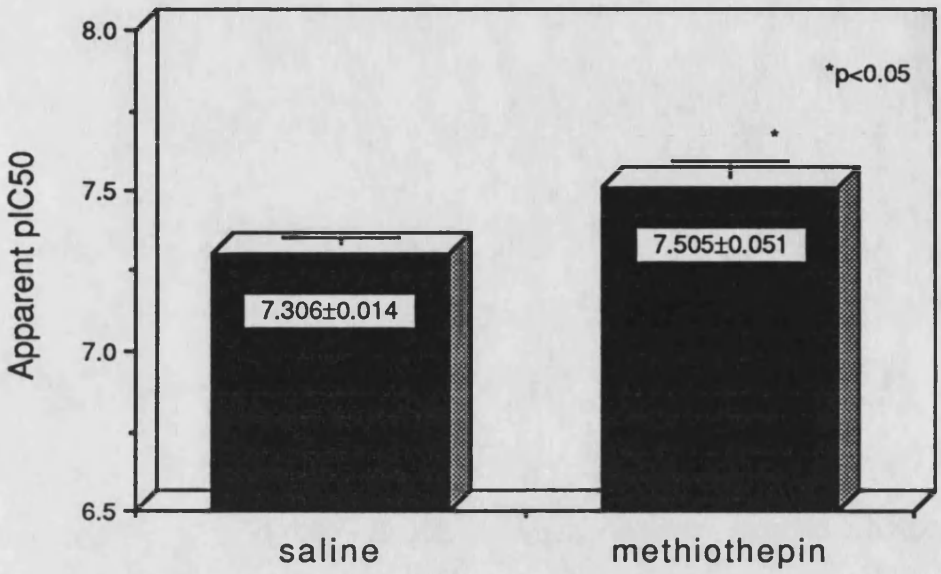
2.4.10 Timing of experiments

Little is known about the seasonal variation in the sensitivity of the terminal 5-HT autoreceptor. This area was not investigated, but the timing of the various experiments is provided so that it may be of use to experimenters wishing to investigate for seasonal variations in terminal 5-HT autoreceptor sensitivity.

The release studies from rat cerebral cortex slices using 5-HT and methiothepin were performed in march/april and april/may respectively. The release studies from rat hippocampal slices were performed in october/november (5-HT) and january (methiothepin). The release studies from rat hippocampal slices using noradrenaline were performed in january/february, while the antagonist studies using phentolamine were carried out in april/may.

The agonist (5-HT) and the antagonist (methiothepin) studies using guinea-pig cerebral cortex slices were carried out in july/august.

Figure 25. Apparent pIC_{50} values for inhibition of the release of [^{3}H]5-HT from slices of rat cerebral cortex by 5-HT, following the chronic administration of methiothepin



The rats in each group received daily injections of either saline or methiothepin (10 mg/kg i.p.) for 14 days. Three days after the last injection the rats were killed and the sensitivity of the terminal 5-HT autoreceptor determined, by the ability of exogenous 5-HT to inhibit the release of previously taken up [^{3}H]5-HT. The results are the means ± s.e.m. of three separate experiments for each group.

2.5 DISCUSSION

A circadian variation in the concentration of 5-HT in rodent brain is well established (Quay, 1968 ; Hillier and Redfern, 1976b). Although the phase of the rhythms of 5-HT concentrations is not identical in all brain regions, the peak concentrations occur during the light phase (Hery *et al.*, 1972). This variation in neuronal 5-HT concentration is inversely related to the release of 5-HT, which is greatest during the dark phase (Martin and Marsden, 1985), when the firing of 5-HT neurones is highest and the animals are most active (Kalen *et al.*, 1989). The release of 5-HT is, in turn, affected by many factors, including the activity of the terminal 5-HT autoreceptors which, when stimulated by 5-HT, inhibit transmitter release by a negative feedback mechanism (Moret, 1985). It was proposed recently that this 5-HT autoreceptor exhibits a circadian variation in sensitivity (Martin *et al.*, 1987) which may account for the circadian variation in 5-HT release and also for the variation in 5-HT concentrations. The aim of this study was to investigate the sensitivity of this 5-HT autoreceptor during the light : dark cycle.

The terminal 5-HT autoreceptor has been identified in a number of regions of the rat brain, where it has been correlated with the 5-HT_{1B} binding site (Engel *et al.*, 1986), while in the guinea-pig this 5-HT autoreceptor is shown to correspond to the 5-HT_{1D} binding site (Hoyer and Middlemiss, 1989)(see section 1.4).

Functional evidence for this classification in the rat has been provided by the weak and rather inconsistent effects of the 5-HT_{1A} receptor agonist, 8-OH-DPAT, in inhibition of the potassium-evoked release of tritium from brain slices and synaptosomes (Middlemiss, 1984b ; Engel *et al.*, 1986 ; Maura *et al.*, 1986). These observations are consistent with those of the present study. In contrast, Hamon *et al.* (1984) observed an inhibition of the potassium-evoked release of tritium from cortical and striatal slices by 8-OH-DPAT, an effect which was antagonized by metergoline but not by yohimbine. This inconsistency may be due to the loss of selectivity of 8-OH-DPAT when used at high concentrations. In addition, the lack of effect of another 5-HT_{1A}-selective agent, buspirone (Middlemiss, 1988), further suggests that this 5-HT autoreceptor, modulating 5-HT release, is unlikely to correspond to the 5-HT_{1A} subtype. In contrast, RU 24969, which possesses a high affinity for the 5-HT_{1B} binding site (Sills *et al.*, 1984), is a potent

inhibitor of the release of 5-HT (Gothert *et al.*, 1987).

In the present study, the uptake of [^{3}H]5-HT by cerebral cortex slices of rat brain has been shown to be into serotoninergic neurones by a temperature-dependent mechanism. Other studies have obtained similar results when monitoring the uptake of [^{3}H]5-HT by brain slices and synaptosomes (Shaskan and Snyder, 1970 ; Stauderman and Jones, 1985). Blackburn *et al.* (1967) found that the majority of the [^{3}H]5-HT taken up by brain slices could be recovered from subsequently-prepared synaptosomes, suggesting that the process/es responsible for this uptake is/are located on 5-HT nerve endings.

A circadian variation has been reported in the uptake of [^{3}H]5-HT by slices of rat SCN, with the highest uptake observed during the dark phase (Meyer and Quay, 1976). A variation in 5-HT uptake could lead to an altered release of tritium during stimulation. The circadian variability in the uptake of [^{3}H]5-HT by brain slices was not investigated in the present study, but the results obtained from the potassium-evoked release of tritium and its enhancement caused by methiothepin were very similar at the four time points (for rat cortical, rat hippocampal and guinea-pig cortical slices), suggesting that the uptake is unlikely to be drastically different.

The rat cerebral cortex and hippocampus were chosen for 5-HT autoreceptor sensitivity determination because these regions receive projections from the dorsal raphe and the dorsal and median raphe nuclei respectively (Blier *et al.*, 1990). The superfusion of [^{3}H]5-HT preloaded brain slices with Krebs buffer, containing elevated potassium ions, caused an increase in the efflux of tritium (which was entirely calcium-dependent, suggesting that the release of tritium was from functionally-intact serotoninergic neurones), the magnitude of which was very similar at all four time points, for each region of both species. In contrast, Blier *et al.* (1989a) reported the electrically-evoked release of tritium from rat hypothalamic slices *in vitro* to be lower during the dark phase. These findings are in disagreement with *in vivo* intracerebral dialysis studies where the release of 5-HT from the rat hypothalamus (Martin and Marsden, 1985) and the hippocampus (Kalen *et al.*, 1989) was greater during the dark phase, when the firing rate of 5-HT neurones is highest and the animals are most active (McGinty and Harper, 1976 ; Trulson and Jacobs, 1979). Furthermore, the lower release rate of tritium observed during the dark phase (Blier *et al.*, 1989a) is unrelated to the degree of activation of the 5-HT autoreceptor or

the increased uptake of 5-HT at this time, otherwise the methiothepin-evoked enhancement would have been different and the accumulation of [^{3}H]5-HT would have been greater during incubation. Instead, the variation in the release of tritium is likely to be the result of a mechanism which is independent of the 5-HT autoreceptor (*i.e.* analogous to the increase in the release of tritium following the short-term administration of lithium, with an unaltered 5-HT autoreceptor) (Blier *et al.*, 1987). Moreover, the lack of a variation in tritium release observed in the present study is supported by the similar magnitude of enhancement of tritium release produced by methiothepin.

The virtually identical potassium-evoked release rates observed here throughout the light : dark cycle, suggest that there is also no inherent tendency of 5-HT nerve terminals to release greater amounts of 5-HT during the dark phase, and that the greater release rates seen during the dark phase *in vivo* are probably the consequence of other factors, such as the firing rate of 5-HT neurones.

The cumulative addition of 5-HT caused a dose-related inhibition of the potassium-evoked release of tritium, the dose response curves obtained at each of the four time points being essentially identical, suggesting that the sensitivity of the 5-HT autoreceptor was the same at these times. Similar results were obtained for the rat cortical, rat hippocampal and guinea-pig hippocampal slices. Recently, Blier *et al.* (1989a) have also reported the lack of variation in the sensitivity of the terminal 5-HT autoreceptor of the rat hypothalamus to 5-HT agonists, in slices prepared during the dark and the light phase.

The addition of the putative 5-HT autoreceptor antagonist, methiothepin, to the superfusion buffer produced a significant enhancement of the release of tritium, presumably by blocking the activation of the 5-HT autoreceptor by the released transmitter (Middlemiss, 1984a). The magnitude of this enhancement was similar at each of the four time points, in both the rat cortical and hippocampal slices. Methiothepin also produced an enhancement of the potassium-evoked release of tritium from guinea pig cortical slices, which is in contrast to the findings of Middlemiss *et al.* (1988). This enhancement is likely to be the consequence of the greater endogenous tone created by the use of a higher potassium ion concentration in the present study. The presence of methiothepin in the superfusion buffer resulted in a rightward shift in the dose response curve of 5-HT. The apparent pA_2 values calculated for this shift were not significantly different at the four

time points for the rat cortical and hippocampal slices.

In order to check whether this superfusion technique was capable of detecting sensitivity changes in terminal 5-HT autoreceptors, methiothepin was administered to rats daily for 14 days. This treatment produced a leftward shift of the dose response curve to 5-HT, when compared to control rats. This shift is suggestive of an up-regulation, or increased sensitivity, of the terminal 5-HT autoreceptor to exogenous 5-HT, suggesting that these 5-HT autoreceptors are under a tonic inhibitory influence in the brain. Maura and Raiteri (1984) have reported similar results for sub- and super-sensitive 5-HT autoreceptors in superfused brain synaptosomes following long-term stimulation or blockade respectively, a finding supported by the electrophysiological studies of Chaput *et al.* (1986b) and Blier *et al.* (1988). In contrast, Hagan and Hughes (1983) were unable to detect any changes in 5-HT autoreceptor sensitivity, following chronic methiothepin. This reason for this discrepancy is not clear but it may reflect regional differences.

The changes produced in the sensitivity of the terminal 5-HT autoreceptors by long-term drug administration are likely to be marked changes which may bear little similarity to those expected of an endogenous circadian variation. However, the ability of the terminal 5-HT autoreceptors to exhibit sensitivity changes suggests that they are capable of adapting to their environment and makes it likely that they are in a state of flux, although their inability to change quickly (Hagan and Hughes, 1983 ; Maura and Raiteri, 1984) implies that these changes take some time to become established, thus making it unlikely that they would be produced by the circadian variation in the release of 5-HT. The superfusion technique applied in the present study is capable of detecting the changes in autoreceptor sensitivity induced by the long-term administration of methiothepin, however its ability to detect changes expected of a circadian rhythm will depend on the amplitude of that rhythm, and on the sampling times corresponding to the zenith and nadir of the rhythm.

The release of 5-HT from the serotoninergic terminals of rat brain is also modulated by inhibitory presynaptic *alpha*$_2$-adrenoceptors (Gothert and Huth, 1980 ; Gothert *et al.*, 1981). Activation of these heteroreceptors by exogenous noradrenaline decreases the stimulation evoked release of 5-HT (Gothert and Huth, 1980 ; Maura *et al.*, 1985). If these *alpha*-adrenoceptors are also activated by endogenous noradrenaline, then any alteration in their affinity or functional activity would be reflected as a change in

the release of 5-HT. In support of this concept is the reported circadian variation in the number of *alpha*$_2$-adrenoceptor binding sites in a number of regions of rat brain during the light : dark cycle (Kafka *et al.*, 1986).

The results presented here clearly demonstrate that the sensitivity of the *alpha*$_2$-adrenoceptors regulating 5-HT release in the rat hippocampus, to both agonist and antagonist, is unchanged during the light : dark cycle. In addition, the *alpha*$_2$-adrenoceptor antagonist, phentolamine, failed to enhance the potassium-evoked release of 5-HT at mid-light but did so at mid-dark, thus suggesting that the concentration of endogenously released, noradrenaline that activates this receptor, may be higher at mid-dark. This hypothesis is supported by *in vivo* microdialysis studies of the hippocampus, in which endogenous concentrations of noradrenaline were 43% higher during the dark phase (Kalen *et al.*, 1989). Gothert and Huth (1980) also reported phentolamine to enhance the potassium-evoked release of 5-HT from rat cerebral cortex slices in the absence of a noradrenaline uptake inhibitor. In contrast, phentolamine failed to enhance the stimulation evoked release of 5-HT from rat hypothalamic slices, in the absence or even in the presence of a noradrenaline uptake inhibitor (Galzin *et al.*, 1984). This discrepancy may be due to regional or time-dependent differences in noradrenaline concentrations and implies that the *alpha*$_2$-adrenoceptors present on serotoninergic nerve terminals may play a physiological role in regulating the activity of serotoninergic neurones.

The 5-HT receptor antagonist, methiothepin, exhibits considerable affinity for the *alpha*$_2$-adrenergic and dopaminergic binding sites (Leysen *et al.*, 1981). Its lack of selectivity for the terminal 5-HT autoreceptor suggests that its enhancement of the potassium-evoked release of tritium may be the result of its action on these sites (*i.e.* heteroreceptors regulating 5-HT release). The neurotransmitters that act on these heteroreceptors, themselves display a circadian variation in release. The possibility exists therefore that the circadian variation in 5-HT release may derive from a variation in the activity of one or more heteroreceptors. However, the results presented in this thesis demonstrate that the *alpha*$_2$-adrenoceptor regulating 5-HT release in the rat hippocampus is devoid of a circadian variation in its sensitivity. An investigation into the circadian fluctuation in the sensitivity of the other heteroreceptors and their neurotransmitters needs to be performed. It is likely that factors such as these would

complicate the detection of a circadian variation in 5-HT autoreceptor sensitivity.

The continuous stimulation superfusion technique applied in the present study was analogous to that of Middlemiss (1984a,b) and the results are in excellent agreement with those reported for the rat and the guinea-pig by Middlemiss (1984a,b), Middlemiss *et al.* (1988). This technique has not gained general acceptance. Its unpopularity is attributed to the use of long periods of stimulation of brain slices with elevated potassium ions, an unphysiological condition, although the release during this entire period is calcium-dependent. Comparison with the more conventional S_2/S_1 technique suggests that the two techniques yield similar results (Richards, 1985). Indeed, the continuous stimulation technique possesses the advantage of "enabling cumulative dose-response curves to 5-HT autoreceptor agonists to be constructed within one experiment, thereby reducing variability and increasing productivity" (Middlemiss, 1988). Although the S_2/S_1 technique does allow each slice or synaptosomal preparation to act as its own control, thereby reducing variability, this advantage is offset by the need to carry out at least 6-8 experiments at each drug concentration to ensure adequate reproducibility.

Superfusion studies of brain slices are generally performed in the presence of a selective 5-HT uptake inhibitor, to prevent the reuptake of transmitter and the displacement of [^{3}H]5-HT from its storage sites. An uptake inhibitor is also necessary to achieve detectable and stable release rates (Gothert, 1980). The inclusion of uptake inhibitors has also been shown to attenuate the inhibitory effect of agonists at the 5-HT autoreceptor and the *alpha*$_2$-adrenoceptor by means of the proposed functional link (Gothert *et al.*, 1983 ; Galzin *et al.*, 1985). In addition, uptake inhibitors can change the biophase concentration of the liberated transmitter by raising its extracellular concentrations. The extent to which this phenomena contributed to the results obtained in this thesis is not known, but they could account for the discrepancies between functional potencies (pIC_{50}) or apparent pA_2 values, and the pK_d values from binding experiments which are performed in the absence of an uptake inhibitor.

A low frequency stimulation technique has been described recently which circumvents this problem. It involves the superfusion of brain slices in the absence of a 5-HT uptake inhibitor, and yields apparent pIC_{50} values which are closer to the pK_D values of binding studies (Blier *et al.*, 1989b). Stimulation is performed using a frequency which

corresponds to the firing rate of 5-HT neurones (1-3 Hz), producing low concentrations of endogenous 5-HT in the synaptic cleft and thus minimizing feedback inhibition. The apparent pIC_{50} value for the inhibitory effect of the putative 5-HT autoreceptor agonist, 5-methoxytryptamine is sixteen times lower at 1 Hz than at 3 Hz, suggesting a lesser degree of activation of the 5-HT autoreceptor. Also, the putative 5-HT autoreceptor antagonist, methiothepin, produces a greater enhancement of the release of tritium at 3 Hz than at 1 Hz. This technique may prove useful in the detection of rhythms of small amplitude (*i.e.* smaller changes in autoreceptor sensitivity). It would interesting to use this technique to determine the sensitivity of the hypothalamic terminal 5-HT autoreceptor, during the light : dark cycle, since this region contains the primary endogenous pacemaker which is responsible for the regulation of circadian rhythms of several functions.

As yet there are no suitable behavioural models for assessing the activity of the terminal 5-HT autoreceptor, although the hyperlocomotion observed following the administration of RU 24969 was attributed to the activation of the $5\text{-}HT_{1B}$ receptors (Green *et al.*, 1984), since RU 24969 exhibits considerable affinity and selectivity for the $5\text{-}HT_{1B}$ binding site (Sills *et al.*, 1984). Examination of the effect of RU 24969 during the light : dark cycle did not reveal any evidence of a significant 24 hour variation (Moser, 1986). In contrast, Martin *et al.* (1987) reported a variation in the ability of metergoline to antagonize the RU 24969-induced hyperlocomotion, metergoline being three to five times more effective during the light phase in comparison to the dark phase. These authors suggested that this difference, in effect, was the result of a circadian variation in the sensitivity of the terminal 5-HT autoreceptor. However, the link between the RU 24969-induced hyperlocomotion and the involvement of a 5-HT receptor has been questioned on the grounds of the ineffectiveness of a number of 5-HT antagonists (Green *et al.*, 1984 ; Tricklebank *et al.*, 1986). Also, this behavioural effect of RU 24969 cannot be attributed to the activation of a presynaptic 5-HT receptor because it was not affected by PCPA depletion (Green *et al.*, 1984) or by the intracerebroventricular injection of 5,7-DHT (Tricklebank *et al.*, 1986). In addition, the RU 24969-induced hyperlocomotion can be blocked by haloperidol or prazosin, suggesting a catecholamine involvement in this behavioural effect (Tricklebank *et al.*, 1986). The changes in autoreceptor sensitivity proposed by Martin *et al.* (1987) may be secondary to a variation in pharmacokinetic

handling and/or the dopamine antagonist activity of metergoline.

On the basis of the greater selectivity of RU 24969 for the 5-HT_{1B} binding site, it was proposed recently that this receptor may mediate hypophagia (Kennett *et al.*, 1987). This hypophagic effect of RU 24969 is considered to be mediated by a postsynaptic 5-HT_1 receptor (Kennett *et al.*, 1987 ; Hutson *et al.*, 1988 ; Kennett and Curzon, 1988). In addition, the hypophagic effect is not secondary to the hyperlocomotion, since haloperidol blocks the hyperlocomotion but not the hypophagia, whereas metergoline blocks the hypophagia (Kennett *et al.*, 1987). Furthermore, the direct infusion of RU 24969 into the paraventricular nucleus of the hypothalamus produced hypophagia with no change in locomotor activity, whereas 8-OH-DPAT was devoid of any activity, suggesting that the effect of RU 24969 is likely to be mediated by a non-5-HT_{1A} receptor, possibly 5-HT_{1B} (Hutson *et al.*, 1988). A circadian variation in this hypophagic response of RU 24969 has not been investigated.

It was reported recently by Clark *et al.* (1988), that considerably lower doses of RU 24969 than those used in the behavioural studies described above produced hypolocomotion, which was secondary to a sedative effect, and mediated by the presynaptic 5-HT_{1B} autoreceptor. However, studies performed in our laboratory (not reported here) to repeat this work and to investigate the existence of a circadian variation in this behavioural effect were unsuccessful.

The question that needs to be addressed is, what is the exact functional role of the terminal 5-HT autoreceptors ? Their location on 5-HT nerve terminals is ideal for enabling them to modulate neurotransmitter release. The neurotransmitter released into the synaptic cleft activates not only the post-synaptic 5-HT receptors but also the presynaptic 5-HT autoreceptors which act *via* negative feedback to inhibit further release of the transmitter. At the same time, the majority of the released transmitter is taken back up from the synaptic cleft into 5-HT nerve terminals by the 5-HT uptake mechanism. The combined effect serves to reduce the concentrations of transmitter in the synaptic cleft, thereby preparing the system for the arrival of the next action potential. In this way, each presynaptic depolarization would be expected to produce a corresponding clear-cut activation of the postsynaptic 5-HT receptor. On this basis, the presynaptic 5-HT autoreceptor functions to make neurotransmission not only more efficient but also more

precise.

The results of the present *in vitro* study demonstrate that the sensitivity of rat cortical and hippocampal terminal 5-HT_{1B} autoreceptors, to both agonist and antagonist, is unchanged during the light : dark cycle. Similarly, the sensitivity of the guinea-pig cortical, terminal 5-HT_{1D} autoreceptor to the agonist is the same during the light : dark cycle. The inability to detect changes in terminal 5-HT autoreceptor sensitivity, in either the rat or the guinea-pig, suggests that these receptors intrinsically lack circadian activity. It is therefore unlikely that they would be directly responsible for the observed circadian variation in the synthesis, release or the metabolism of 5-HT, although their activity would be susceptible to modulation by the changes in 5-HT concentration in the synaptic cleft. In addition, the low affinity of noradrenaline for the *alpha*$_2$-adrenoceptors located on 5-HT nerve terminals, and their unchanged sensitivity over 24 hours also makes their direct responsibility for the circadian rhythm in 5-HT concentrations unlikely. Furthermore, the circadian rhythm of noradrenaline release, which is greater during the dark period, would serve to dampen the rhythm in 5-HT release.

In conclusion, the continuous stimulation technique is capable of detecting drug-induced changes in terminal 5-HT autoreceptor sensitivity, but these receptors were devoid of an innate variation in their sensitivity to agonist or antagonist, during the light : dark cycle. The failure to observe a circadian variation in their sensitivity may be due to its absence, as suggested by the results presented here or the inability of this technique to detect them. It is also possible that this same class of terminal 5-HT autoreceptors could display a circadian variation in their sensitivity, when examined in other brain regions. It is likely therefore that factors other than any circadian rhythm, in terminal 5-HT autoreceptor or *alpha*$_2$-adrenoceptor function, must be assumed to control the observed circadian rhythm in 5-HT release.

* The detection of a circadian rhythm in 5-HT autoreceptor sensitivity may be hampered by the experimental protocol employed, since this superfusion technique measures autoreceptor sensitivity during the course of the experiment (*i.e.* over 118 min.), it may not allow for the detection of acute changes which could take place during the course of the experiment.

3 RADIO-LIGAND BINDING STUDIES

3.1 METHODS FOR [^{3}H]5-HT binding

3.1.1 Preparation of membranes

The procedure of membrane preparation described by Herrick-Davis and Titeler (1988), was used with minor modifications. Six male Wistar rats (University of Bath strain, 200-350g), were killed by cervical dislocation in either the dark or the light phase as appropriate. The animals killed in the dark phase had their eyes enucleated prior to them being brought out into the light for dissection. The brains were then rapidly removed and placed on an ice-cooled petri-dish. The cerebral cortex was then dissected. The tissue pooled (approx. 2.5 g wet weight) and homogenized (Ultra Turrax, 1/3 maximum speed for 30 seconds) in ice-cold buffer (composition : 50 mM Tris-HCl, 0.5 mM EDTA and 10 mM $MgSO_4$, p.H. 7.4 (1:10 wt/vol). The homogenate was further homogenized using a teflon mortar and pestle (Tri-R Stir-R, model K 41), four slow up and down strokes at 1/3 speed. The homogenate was then centrifuged (Beckman Ultracentrifuge, model L8-M) at 30,000 x g for 15 minutes. The resulting pellet was resuspended and homogenized (Ultra Turrax for 30 sec.) in ice-cold distilled water, to disrupt nerve endings containing endogenous 5-HT (1:30 wt/vol, p.H. 7.4), and recentrifuged for 10 minutes. The subsequent pellet was this time resuspended and homogenized (Ultra Turrax for 30 sec.) in Tris-HCl buffer (1:30 wt/vol), incubated at 37°C (Grant, model JB 2) for 15 minutes and centrifuged a further four times, 10 minutes each, with a resuspension between centrifugations. The pellet was suspended once more in distilled water preceding the fourth centrifugation. The final pellet was resuspended in buffer containing 50 mM Tris-HCl, 0.5 mM EDTA, 10 mM $MgSO_4$, 0.1% ascorbic acid and 10 µM pargyline. Some membranes were frozen overnight in liquid nitrogen (-195°C).

The method described above was also used to prepare membranes from male hamster and guinea-pig cerebral cortex. Due to the smaller size of the hamster brain, six animals were killed for each experiment and their cerebral cortices pooled. In contrast, the tissue obtained from a single guinea-pig was sufficient for each experiment.

3.1.2 Purity of [^{3}H]5-HT

It is essential to ensure that the [^{3}H]5-HT used in the binding studies is not only

pure but does not degrade during the course of the experiment, as this would cause an underestimation of the equilibrium dissociation constant. In addition, any product formed as a result of the degradation of the ligand possesses the potential to interfere with the binding assay. The results of such studies can be misleading, as the breakdown product may well contribute to the binding of the ligand.

It is essential therefore to identify any potential hazards which are likely to interfere with the assay, and if possible eliminate them. [^{3}H]5-HT is prone to oxidation, therefore the inclusion of an antioxidant (ascorbic acid 0.1%), is commonly employed to reduce this potential. 5-HT is metabolized to 5-hydroxyindole acetic acid (5-HIAA) by the action of monoamine oxidase (MAO). The inclusion of pargyline (10 μM), which is an irreversible MAO-inhibitor, reduces the likelihood of this happening to [^{3}H]5-HT.

The [^{3}H]5-HT when used was 97.7% pure, and all the necessary binding experiments were performed within one month of its receipt. In addition, some of the preliminary work was carried out using an older batch of [^{3}H]5-HT which was only 48% pure, as assayed by HPLC linked to a electrochemical detector.

3.1.3 Investigation of the serial dilution of [^{3}H]5-HT concentrations

Serial dilutions of [^{3}H]5-HT were carried out in 20 ml plastic scintillation vials. Six standard concentrations of [^{3}H]5-HT were prepared in Tris-HCl buffer and used in the radioligand binding studies described. The standards being prepared immediately prior to each experiment.

3.1.4 Assessment of the degree of [^{3}H]5-HT binding to filter paper

The extent of [^{3}H]5-HT binding to Whatman GF/B filter paper was investigated. This type of binding is an example of non-specific binding which can sometimes obscure the specific component. It is therefore imperative that all forms of non-specific binding be kept to a minimum.

The filters underwent a variety of treatments :

(a) soaked in Tris-HCl ;

(b) soaked in a 10 μM solution of 5-HT ;

(c) soaked in a 0.1% solution of polyethyleneimine ;

(d) kept dry.

A concentration of [^{3}H]5-HT approximating to its dissociation constant (K_D) value (2 nM) was chosen to pass through the filters. A volume of 1 ml was passed through each filter, followed by two 5 ml washes with ice-cold Tris-HCl buffer. The filters were then placed into plastic scintillation vials and 12 ml of liquid scintillation fluid added. Following sonication for 15 minutes they were allowed to equilibrate in the scintillation counter for three hours, prior to counting for 5 or 10 min each. The results were expressed as disintegrations per minute.

3.1.5 Protein assay

A 1:10 dilution of the final membrane suspension was made and this was used for the estimation according to the method of Lowry *et al.* (1951). Standard concentrations of bovine serum albumin were prepared ranging from 10-60 μg.

Stock solutions of 2% (w/v) Na_2CO_3 in 0.1 M NaOH, 1% (w/v) $CuSO_4$ and 2% (w/v) Sodium tartrate were made and stored at 4°C. They were mixed in a ratio of 100:1:1 immediately prior to the assay. A 100 μl sample diluted to 200 μl with buffer or in the case of standards the appropriate volume being made up to 200 μl with buffer. One ml of the above prepared mixture was added to all the tubes. They were whirlimixed and left at room temperature for 10 min. After this period, 100 μl of 50% Folin's reagent was added to all the tubes. The tubes were again whirlimixed and left at room temperature for 40 min, for the blue colour to develop. The absorbance at 750 nM was measured using a Pye Unicam PU 8610 UV spectrophotometer. All standards were run in duplicates, while the samples were run in triplicate. The protein concentration was determined graphically from the linear range of the standard curve.

3.1.6 Investigation of Washing

The binding of a radioligand to a single set of sites, displaying the law of mass action kinetics, is completely described by the two parameters K_D and B_{max}.

The dissociation constant calculated experimentally should theoretically be independent of the membrane concentration, so long as the bound and free ligand have been carefully quantified. However, the protein concentration can influence the K_D value (Leysen and Gommeren, 1981). Furthermore, non-specific binding and the dissociation constant have been reported to double when the protein concentration is increased four fold (Lazareno and Nahorski, 1982). This problem was attributed to the inefficient washing of the membranes and can be minimized if a lower protein concentration is used, in conjunction with adequate washing.

Washing of the membranes was therefore investigated experimentally. Unfortunately, the washing process is a compromise between removing as much unbound or non-specifically bound ligand as possible, whilst causing little dissociation of the specifically bound ligand. Practically, it is best to use a copious amount of buffer in as shorter time as possible.

The following protocol was employed. A 1 ml suspension of membranes (containing 0.5 mg protein) was incubated at 37°C together with [^{3}H]5-HT at a concentration of 2 nM, for 30 min. After this period of incubation the membranes were filtered through Whatman GF/B filters followed by either two or three 5 ml washes with ice-cold Tris-HCl buffer.

3.1.7 Investigation of the specific binding of [^{3}H]5-HT at varying protein concentrations

As reported in the section 3.1.6, the concentration of protein used can have a marked influence on the parameters being determined. It is therefore important to use a concentration of protein which is in the range at which the degree of binding shows a good linear relationship with the amount of membrane protein. This has the effect of minimizing problems which could occur as a result of receptor or ligand degradation during the incubation or uneven washing of the free radioligand as the protein concentration varies.

An investigation was carried out into the effect of varying concentrations of protein on specific binding, and the ability of the filter paper to cope with this amount of protein without getting blocked. Protein concentrations between 0.2 and 1.0 mg per

incubation were used and binding was assessed using approximately 2 nM of [^{3}H]5-HT. Non-specific binding was that measured in the presence of 10 μM 5-HT.

3.1.8 Investigation of protein loss through filters

It is important to establish that the process of membrane washing does not result in the loss of protein through filters, as this is likely to lead to an underestimation of the amount of [^{3}H]5-HT bound per mg protein.

To establish whether protein was being lost through the filter, incubations were carried out as previously described. They were filtered through Whatman GF/B filters followed by one or two 5 ml washes with ice-cold Tris-HCl buffer. The filtrate was collected in test-tubes and the protein was estimated by the method of Lowry *et al.* (1951).

3.1.9 Specificity of [^{3}H]5-HT binding to membranes

Some binding studies have been plagued with the binding of the ligand to rather obscure and unsuspected sites. Bielkiewicz and Cook (1985), reported the specific, high affinity binding of the histamine receptor ligand, mepyramine, to glass fibre filters. Peroutka and Demopulos (1986), reported similarly the specificity of the binding of 8-OH-DPAT to glass fibre filter papers. The binding of radiolabelled insulin to talc is another classic example (Cuatrecasas and Hollenberg., 1975). It is therefore imperative that necessary steps are taken to ensure that the ligand binding to these non-specific sites is kept to a minimum, or it may obscure the view of the physiologically relevant sites.

In order to establish that the ligand does in fact bind to the membranes being used, the membranes (0.25 mg protein) were heated to boiling in order to denature the receptor proteins. The binding procedure was then carried out in the usual way. The amount of binding detectable was very low (total=208±19 ; non-specific=217±10 (mean±s.e.m., n=2)) and was of the order usually seen in the presence of a large excess of 5-HT (*i.e.* non-specific binding). This implies that denaturation of the receptor protein leads to a significant reduction in ligand binding, approximating to non-specific levels.

In addition, the number of counts produced when no ligand was used in the incubation

(*i.e.* when membranes were filtered on their own and the samples counted) was also determined. The number of counts produced was very low and in line with those observed during background estimates. Also, when 2 nM [^{3}H]5-HT, 100nM 8-OH-DPAT and 1 μM (-)propranolol were incubated without membranes, to determine the extent of filter binding, no specific binding was detected. These findings suggest that the ligand binds to membranes and not to the filter.

A further experiment was performed in which membranes (0.5 mg protein in 0.8 ml), ligand (2 nM [^{3}H]5-HT), 5-HT (10 μM) and buffer were used in a number of combinations to further establish the specificity of binding :

(a) 0.8 ml membranes + 0.1 ml Tris-HCl + 0.1 ml ligand ;

(b) 0.8 ml membranes + 0.1 ml 5-HT + 0.1 ml ligand ;

(c) 0.8 ml Tris-HCl + 0.1 ml Tris-HCl + 0.1 ml ligand ;

(d) 0.8 ml Tris-HCl + 0.1 ml 5-HT + 0.1 ml ligand.

3.1.10 [^{3}H]5-HT binding to rat cerebral cortex membranes over 24-hours

The procedure for the binding assay of [^{3}H]5-HT was adapted from Herrick-Davis and Titeler (1988). Saturation experiments were performed using polystyrene tubes in duplicate with a final volume of 1.0 ml [0.8 ml membrane homogenate (containing 0.5 mg protein), 0.1 ml radioactive ligand and 0.1 ml of the appropriate concentration of drug or buffer]. Six concentrations of [^{3}H]5-HT (0.5-16 nM) were used to determine total binding. Non-specific binding was defined as that measured in the presence of 10 μM 5-HT. Competition experiments were performed in triplicate using 2 nM [^{3}H]5-HT as the ligand concentration. The competing drugs used were 8-OH-DPAT (100 nM) and (-)propranolol (1 μM). All assay tubes were incubated at 37°C in a water bath (Grant, model JB 2), for 30 minutes. Incubations were then rapidly terminated by vacuum filtration through Whatman GF/B filters (presoaked in a 0.1% solution of polyethyleneimine for approx. 18 hours), followed by two 5 ml washes with cold buffer, using a Millipore sampling manifold (model No. 1225). The complete washing procedure took approximately 12 seconds per filter. Filters were placed in plastic scintillation vials containing 10 ml of liquid scintillation fluid

(Optiphase "Safe"). The vials were then sonicated (Dawe ultrasonics) for 15 minutes and following vigorous shaking they were left in an LKB liquid scintillation counter for 3 hours to equilibrate. After this period, they were counted at an efficiency of 37.5% efficiency, for 5 minutes. The scintillation counter corrected for the background counts as well as the low efficiency of counting and expressed the results as disintegrations per minute, these were converted to fmol/mg protein.

3.1.11 [^{3}H]5-HT binding to cerebral cortex membranes of hamster and guinea-pig

Saturation and competition studies were performed in the same way as that described above for the binding of [^{3}H]5-HT to rat cerebral cortex membranes.

3.1.12 Effect of chronic methiothepin on [^{3}H]5-HT binding

Methiothepin was administered to rats on a chronic basis, as described in section 2.3.10. Rats were killed by cervical dislocation 3 days after the last injection. The cerebral cortex from five male rats which had previously received either methiothepin or saline injections was dissected and pooled separately. Membranes were prepared as described in section 3.1.1. Competition experiments were performed in triplicate using 2 nM [^{3}H]5-HT as described in section 3.1.10.

3.1.13 Calculations and Statistics

The binding data of saturation studies with both ligands was assumed to fit a one-site model. It was analysed using the program of Tallarida and Murray operated on a Superbrain II computer. This Scatchard analysis program enabled the estimation of the correlation coefficient of binding (r), the equilibrium dissociation constant (K_D) and the number of binding sites (B_{max}).

The one-way analysis of variance test was used for comparison of K_D and B_{max} values for the 5-HT_1 binding site as well as the reduction in the binding caused by the presence of 8-OH-DPAT or (-)propranolol.

3.1.14 Drugs

[^{3}H]5-HT, 12.5-13.5 Ci/mmol (Amersham Int., Amersham, England), 5-hydroxytryptamine creatnine sulphate (Sigma, St. Louis, MO, USA), 8-hydroxy-2-(di-n-propylamino) tetralin (Semat Technical Ltd., St. Albans, England), methiothepin maleate (gift from Roche, Herts, England), pargyline hydrochloride (Sigma), polyethyleneimine (Sigma) and (-) laevopropranolol hydrochloride (gift from ICI, Cheshire, England).

3.2 RESULTS OF [^{3}H]5-HT binding studies

3.2.1 Investigation of the serial dilution of [^{3}H]5-HT concentrations

Six different concentrations (5, 10, 20, 40, 80 and 160 nM) of [^{3}H]5-HT were prepared by serial dilution in plastic scintillation vials using Tris-HCl buffer of the composition used to suspend the final pellet. These concentrations were ten fold higher than those used in binding studies because a further 1:10 dilution would occur when 100 µl of these solutions was added to 900 µl of membranes/drug. A 100 µl solution of each of these concentrations was counted in triplicate. A good correlation was seen when D.P.M. counts were plotted against [^{3}H]5-HT concentration (Fig. 26), suggesting that pipetting was satisfactory and that [^{3}H]5-HT dissolution was uniform.

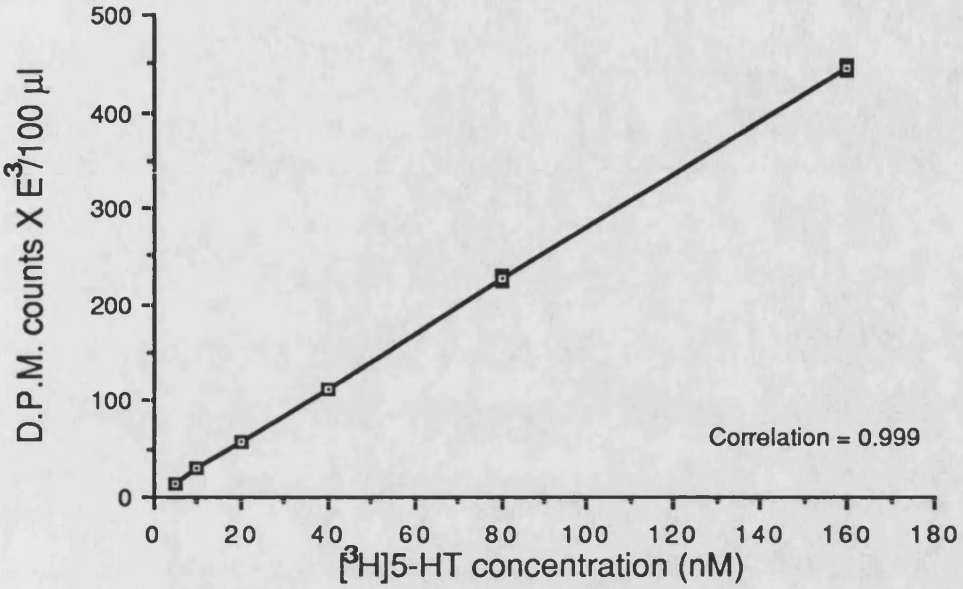
3.2.2 Extent of [^{3}H]5-HT binding to filter paper

Soaking of filter papers (in Tris-HCl, 5-HT or polyethyleneimine) was associated with a reduction in the binding of [^{3}H]5-HT as compared to dry filters. The greatest reduction in this form of non-specific binding was produced in filters previously soaked in either 10 µM 5-HT or 0.1% polyethyleneimine (Table 7). As a result, filters were soaking routinely in a solution of 0.1% polyethyleneimine for at least 12 hours prior to use.

3.2.3 Investigation of washing

Rat cerebral cortex membranes (0.5 mg protein) were incubated at 37°C with [^{3}H]5-HT (2 nM) for 30 minutes. The membranes were then filtered on GF/B filters and washed either twice or three times with 5 ml ice-cold Tris-HCl buffer. Washing with less than a total volume of 10 ml was not tested on the grounds that it may not be enough to

Figure 26. Effect of the serial dilution of [^{3}H]5-HT against D.P.M. counts



The serial dilutions were performed in plastic scintillation vials using Tris-HCl buffer of the composition described in the methods. The results are the means±s.e.m of two separate experiments each performed in triplicate.

wash away unbound ligand. There was little difference between the counts remaining after washing either twice or three times with 5 ml buffer (Table 8). From this study, a washing procedure involving two 5 ml washes with ice-cold buffer was chosen for subsequent studies.

3.2.4 Specific binding of [^{3}H]5-HT at varying protein concentrations

Increasing protein concentration in the incubation was associated with a linear increase in the total and the specific binding components, up to about 0.6 mg protein (Fig. 27). In contrast, non-specific binding increased linearly with protein concentration. The filter was blocked when 1.0 mg protein was filtered. From this study, a membrane protein concentration of 0.5 mg was chosen for future work on the grounds that it falls on the linear range of the specific binding line and also because higher protein concentrations yield higher binding counts.

3.2.5 Investigation of protein loss through filters

No protein was detected in the eluate when the filters were washed twice with 5 ml Tris-HCl buffer. As a further check, the membranes were washed with just 2 ml of ice-cold Tris-HCl buffer. It was found that approximately 4.14% ± 0.72% (n=4) of the protein used (0.5 mg per incubation), was recovered in the filtrate. This small amount of protein was undetectable when the membranes were washed with either 5 or 10 ml of buffer, perhaps because of the dilution effect. This filtered protein is likely to consist of small membrane fragments and/or small intracellular synaptic components. While this loss is undesirable, it is acceptable.

3.2.6 Specificity of [^{3}H]5-HT binding to membranes

The absence of membranes in the incubation (replaced with 0.8 ml Tris-HCl buffer) was associated with a marked reduction in the total and non-specific binding of [^{3}H]5-HT (Table 9), suggesting that the ligand was binding to membranes. In addition, the denaturation of membrane proteins resulted in a lowering of the binding of [^{3}H]5-HT.

Table 7. Effects of various treatments on the binding of [^{3}H]5-HT to filter paper

Treatment	D.P.M. counts (mean±s.e.m.)	
soaked in Tris-HCl	84.83±5.76	(n=6)
soaked in 10 µM 5-HT	60.25±3.04	(n=12)
soaked in 0.1% polyethyleneimine	61.92±4.01	(n=12)
kept dry	107±10.69	(n=4)

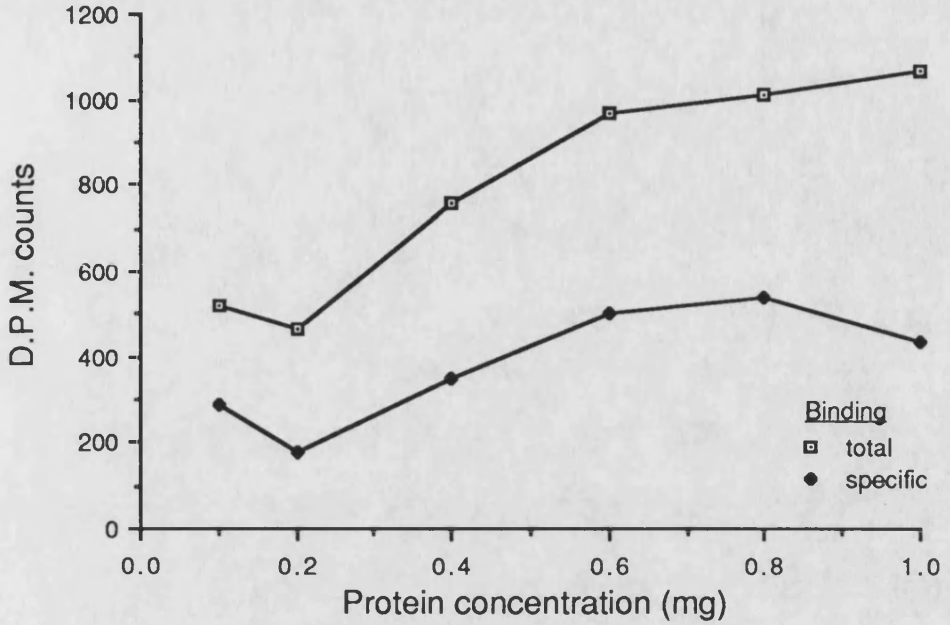
The filter papers were soaked in the appropriate solution for at least least 4 hours, after which 1 ml of 2 nM [^{3}H]5-HT was filtered, followed by two 5 ml washes with ice-cold Tris-HCl buffer. The counts represent the means ± s.e.m. of [^{3}H]5-HT bound to the filters.

Table 8. Effect of the number of washes on the total binding of [^{3}H]5-HT

volume	number of washes	time taken (seconds)	D.P.M. counts (mean±s.e.m.)
5 ml	2	12	2083±53.57
5 ml	3	17	2043±55.39

Rat cortical membranes (0.5 mg protein) were incubated at 37°C with [^{3}H]5-HT at a concentration of 2 nM, for 30 min. The membranes were then filtered and washed with either two or three 5 ml of ice-cold Tris-HCl buffer. The counts are the means ± s.e.m. of determinations performed in triplicate, from a single experiment.

Figure 27. Effect of varying protein concentrations on the total and specific binding of [^{3}H]5-HT



The results are presented as mean D.P.M. counts of a single experiment performed in duplicate. Total binding was measured using approximately 2 nM [^{3}H]5-HT and non-specific binding was that present after the addition of 10 μM unlabelled 5-HT. Specific binding was calculated by subtracting non-specific binding from the total binding.

Table 9. Specificity of [^{3}H]5-HT binding to membranes of rat cerebral cortex

	0.8 ml membranes 0.1 ml [^{3}H]5-HT	0.8 ml Tris-HCl 0.1 ml [^{3}H]5-HT
0.1 ml 5-HT :	304	141
0.1 ml Tris-HCl :	1056	133

Results are the mean D.P.M. counts of a single experiment performed in triplicate. Incubations were performed in a number of combinations using membranes (0.5 mg protein in 0.8 ml), 0.1 ml [^{3}H]5-HT (2 nM final conc.), 0.1 ml Tris-HCl (50 mM) and 5-HT (10 µM final conc.), for 30 minutes and then filtered on GF/B filters. The results are expressed as the counts remaining following two washes with 5 ml ice-cold Tris-HCl buffer.

These results add further support to the idea that [^{3}H]5-HT was binding to a protein component of membranes.

3.2.7 [^{3}H]5-HT binding to cerebral cortex membranes over-24 hours

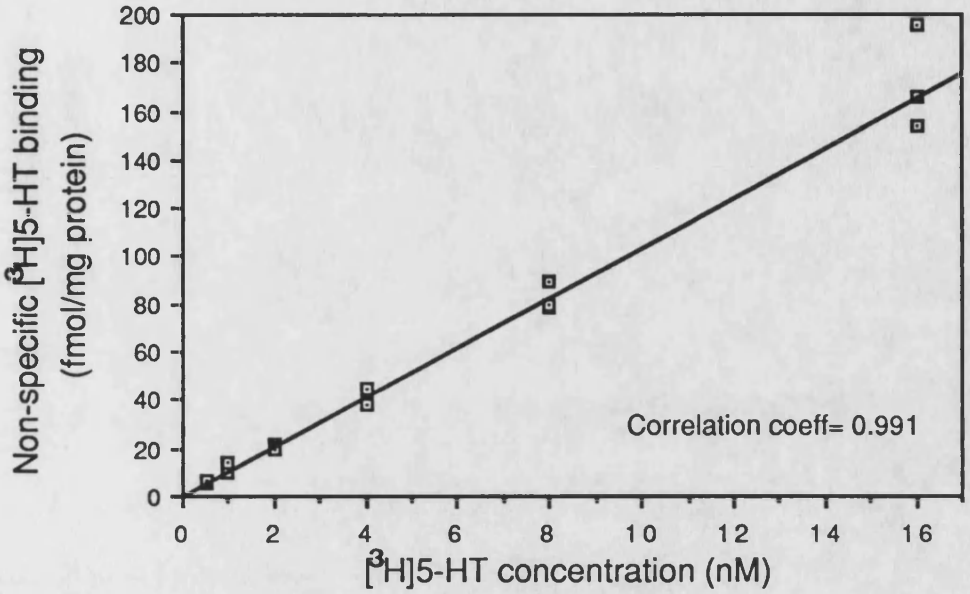
Saturation studies were performed at each time point (mid-dark, mid-light, end-dark and end-light) using six different concentration of [^{3}H]5-HT (0.5 to 16 nM). Non-specific binding was defined as that measured in the presence of 10 μM 5-HT. A plot of non-specific [^{3}H]5-HT binding against [^{3}H]5-HT concentration produced a straight line with a correlation value of 0.991, as analysed by linear regression (Fig. 28). The good correlation confirms that the washing procedure removed all the washable non-specific binding at all concentrations of [^{3}H]5-HT. A similar linear relationship of non-specific binding was also seen at the other three time points.

The specific binding of [^{3}H]5-HT, obtained by subtracting non-specific from the total binding, displayed a monophasic saturation curve (Fig. 29), suggesting that it binds to a single site. Similar curves were obtained at all four time points. A Scatchard plot of this data produces a straight line (Fig. 30), further confirming that [^{3}H]5-HT binds to a single site. Similar plots of experiments performed using tissue obtained at the other time points yielded similar results: high reproducibility between independent experiments and straight Scatchard plots. K_D and B_{max} values were calculated from Scatchard plots for each experiment by regression analysis. In general the specific binding represented about 82% of total binding, K_D and B_{max} values were very similar and not statistically different at the four time points. A summary of the results is shown in Table 10.

A concentration of 2 nM [^{3}H]5-HT (corresponding to the K_D value) was used in competition studies, with specific binding accounting for over 80% of the total binding. 8-OH-DPAT (100 nM) was used to specifically block the 5-HT_{1A} binding sites (Middlemiss and Fozard, 1983), while (-)propranolol (1 μM) is capable of blocking both the 5-HT_{1A} and the 5-HT_{1B} binding sites (Nahorski and Willcocks, 1983 ; Hoyer *et al.*, 1985). The results of this study are shown in Table 11.

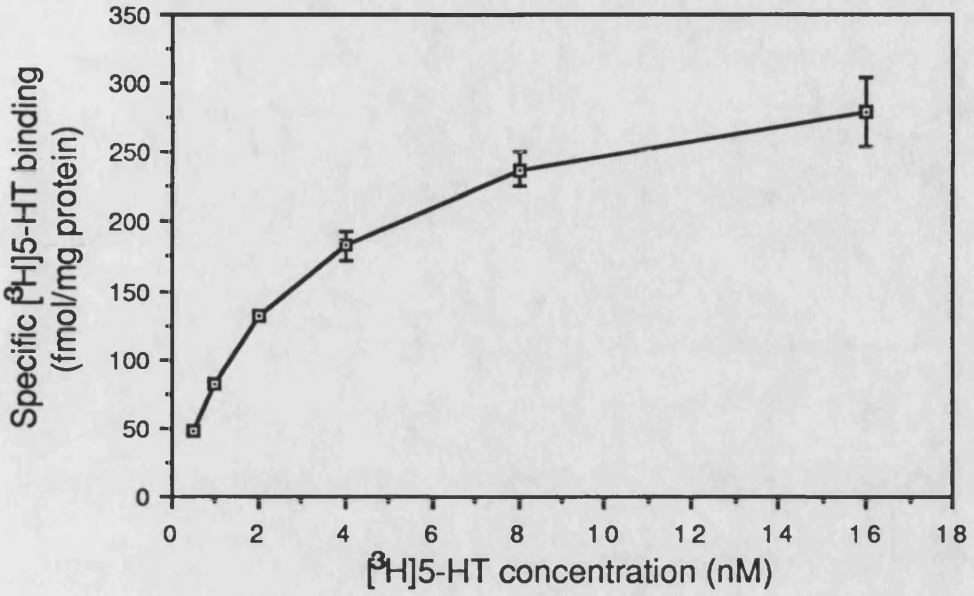
This 2 nM concentration of [^{3}H]5-HT specifically bound to about 120 fmol/mg protein, at each of the four time points. This total probably represents the binding of

Figure 28. Effect of varying concentrations of [^{3}H]5-HT on the non-specific binding component



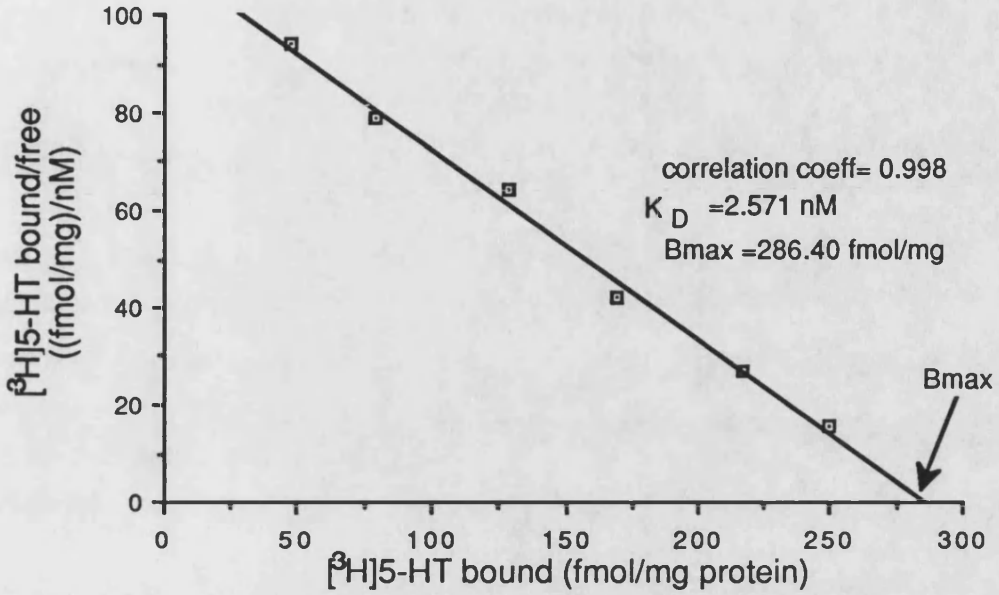
Increasing concentrations of [^{3}H]5-HT (0.5-16 nM) were incubated with membranes (rat cerebral cortex at end-light) in the presence of 10 µM 5-HT, expressed as fmol/mg protein. Each point represents the mean of a duplicate determination, derived from three separate experiments.

Figure 29. The amount of specific [^{3}H]5-HT binding against varying concentrations of [^{3}H]5-HT



Increasing concentrations of [^{3}H]5-HT (0.5-16 nM) were incubated with membranes (rat cerebral cortex at end-light) in the absence or presence of 10 μM 5-HT. Specific binding was calculated by subtracting the non-specific binding from the total binding, expressed as fmol/mg protein. Each point represents the mean ± s.e.m. of three separate experiments each performed in duplicate.

Figure 30. Scatchard plot of the specific binding of [^{3}H]5-HT to membranes of rat cerebral cortex



Scatchard plot of the saturation data of specific [^{3}H]5-HT binding. Specific binding was calculated by subtracting the non-specific binding from the total binding, expressed as fmol/mg protein. This data is from a single assay (rat cerebral cortex at end-light) and is representative of three separate experiments. Each point represents the mean of a duplicate determination.

Table 10. Estimation of the affinity constant and the number of 5-HT_1 binding sites in cerebral cortex membranes of the rat, over 24-hours

	mid-dark	mid-light	end-dark	end-light
K_D (nM)	2.450±0.242	2.992±0.823	2.401±0.161	2.903±0.189
B_{max} (fmol/mg protein)	303±16.29	321±20.94	313±56.65	324±11.69

Specific 5-HT_1 binding was obtained by calculating the difference between total [^{3}H]5-HT binding and binding in the presence of 10 µM 5-HT. Scatchard plots of this data were analysed by linear regression. The affinity constant (K_D) and the number of [^{3}H]5-HT binding sites (B_{max}) were determined for each experiment, at the four time points. Each value represents the mean ± s.e.m. of three separate experiments, each performed in duplicate.

Table 11. Effect of 8-OH-DPAT and propranolol on the binding of [^{3}H]5-HT to rat cortical membranes

site	mid-dark (fmol/mg protein)	% displacement	mid-light (fmol/mg protein)	% displacement
Total 5-HT$_1$	116.18±19.49		119.89±7.82	
8-OH-DPAT	66.31±7.47	43±6.4	64.72±2.66	46±2.2
propranolol	31.77±2.10	73±1.8	33.48±2.65	72±2.2

site	end-dark (fmol/mg protein)	% displacement	end-light (fmol/mg protein)	% displacement
Total 5-HT$_1$	119.74±21.67		127.70±4.61	
8-OH-DPAT	68.29±7.20	43±6.0	72.48±4.36	43±3.4
propranolol	33.81±3.93	72±3.3	35.03±1.44	73±1.1

Competition studies were performed using a 2 nM concentration of [^{3}H]5-HT in the absence or presence of blocking drugs [8-OH-DPAT (100 nM) or (-)Propranolol (1 μM)]. The results are expressed in fmol/mg protein of the [^{3}H]5-HT binding taking place. Each value is the mean ± s.e.m. of three separate experiments at each time point, performed in triplicate.

[^{3}H]5-HT to a range of 5-HT$_1$ subsites. The presence of 8-OH-DPAT (100 nM) in the incubation medium produced a marked reduction (about 43%) in the number of specific [^{3}H]5-HT binding sites, at each of the four time points. This component probably represents the hindrance caused by 8-OH-DPAT in the binding of [^{3}H]5-HT to the 5-HT$_{1A}$ site (Table 11).

(-)Propranolol (1 µM) consistently produced a greater reduction in the binding of [^{3}H]5-HT (representing about 72% displacement of the total), as compared to 8-OH-DPAT. It is likely that this concentration of propranolol interferes partially with the binding of [^{3}H]5-HT to the 5-HT$_{1A}$, 5-HT$_{1B}$ and the 5-HT$_{1C}$ binding sites (Hoyer *et al.*, 1985,1987). As a consequence the results of the use of propranolol are difficult to interpret.

Statistical analysis (one-way analysis of variance) did not identify any significant differences in the affinity of binding, the number of binding sites or the reduction in the binding produced by the presence of drugs.

3.2.8 [^{3}H]5-HT binding to cerebral cortex membranes of hamster and guinea-pig

Saturation studies were performed as described for the rat. Specific binding represented about 84% of total binding in both species. The affinity of [^{3}H]5-HT for these membranes was very similar to that previously observed for the rat. In addition, the B_{max} values were also of similar magnitude (Table 12).

Competition studies performed using 2 nM [^{3}H]5-HT identified a total of 147±11.12 and 132±6.08 fmol/mg protein (mean±s.e.m., n=3) binding sites in hamster and guinea-pig respectively. Of this total, the 5-HT$_{1A}$ binding site accounted for about 45% in hamster and 49% in guinea-pig (Table 12). (-)Propranolol produced a greater reduction of the binding of [^{3}H]5-HT binding as compared to 8-OH-DPAT from hamster cortex membranes, similar to that previously observed in the rat. In contrast, (-)propranolol caused a consistently smaller reduction in the binding of [^{3}H]5-HT binding as compared to 8-OH-DPAT from guinea-pig membranes (Table 12). The inability of propranolol to hinder

Table 12. Extent of [^{3}H]5-HT binding to the 5-HT$_1$ binding site in hamster and guinea-pig cortical membranes

displacing drug	hamster (fmol/mg protein)	% displacement
Total 5-HT$_1$	147.02±11.12	
8-OH-DPAT	80.67±6.79	45±4.6
propranolol	59.27±6.36	60±4.3
K_D (nM)	2.588±0.022	
B_{max} (fmol/mg protein)	344±26.02	

displacing drug	guinea-pig (fmol/mg protein)	% displacement
Total 5-HT$_1$	131.67±6.08	
8-OH-DPAT	66.86±5.36	49±4.1
propranolol	88.54±5.59	33±4.2
K_D (nM)	2.264±0.084	
B_{max} (fmol/mg protein)	292±13.48	

Membranes were prepared from these species at the mid-light time point of their light : dark cycle. Scatchard plots of the saturation data were analysed by linear regression. The affinity constant (K_D) and the number of [^{3}H]5-HT binding sites (B_{max}) were determined for each experiment. Each value represents the mean ± s.e.m. of three separate experiments, each performed in duplicate. Competition studies were performed in the absence or the presence of displacing drugs [8-OH-DPAT (100 nM) or (-)Propranolol (1 μM)], and analysed as described for studies of rat cerebral cortex membranes. The results of competition studies are expressed as the amount of [^{3}H]5-HT binding in the presence or absence of drug, and each value represents the mean ± s.e.m of three separate experiments, each performed in triplicate.

[^{3}H]5-HT binding to a greater extent than that produced by 8-OH-DPAT, suggests that at this concentration it is either incapable or only partially able to prevent [^{3}H]5-HT binding to the 5-HT$_{1A}$ site. In addition, propranolol exhibits considerable affinity for the 5-HT$_{1B}$ binding site in rat brain, however, its inability to yield similar results from guinea-pig cortex membranes suggests that this [^{3}H]5-HT binding site may display an altered affinity for propranolol or that it may be absent.

3.2.9 Effect of chronic methiothepin on [^{3}H]5-HT binding

The results of competition studies from saline-injected animals were very similar to those previously obtained from non-injected rats at this time point. The daily injection of methiothepin (10 mg/kg i.p.) for 14 days produced very little change in the total number of specific [^{3}H]5-HT binding sites {saline=123.52±6.83 ; methiothepin=127.06±3.75 fmol/mg protein (mean±s.e.m., n=3)}. There was no significant difference with respect to control in either the total number of binding sites or the components reduced by 8-OH-DPAT or propranolol (Table 13).

Table 13. Effect of daily methiothepin or saline injections on the number of 5-HT_1 binding sites in the cerebral cortex of the rat

site	saline (fmol/mg protein)	% displacement	methiothepin (fmol/mg protein)	% displacement
Total 5-HT_1	123.52±6.83		127.06±3.75	
8-OH-DPAT	68.22±3.81	45±3.1	73.05±1.11	43±0.9
propranolol	33.31±4.85	73±3.9	35.71±1.96	72±1.5

A group of five rats received daily injections of methiothepin (10 mg/kg i.p.) or saline for 14 days, at the mid-light point of their light : dark cycle. The rats were killed at mid-light, 3 days after the last injection, and the cerebral cortex dissected. Binding studies were performed, following the preparation of membranes, using a 2 nM concentration of [^{3}H]5-HT in either the absence or the presence of blocking drugs [8-OH-DPAT (100 nM) or (-)Propranolol (1 μM)]. The results are expressed as fmol/mg protein of the [^{3}H]5-HT binding taking place. Each value is the mean ± s.e.m. of three separate experiments, each performed in triplicate.

3.3 METHODS FOR [^{3}H]Ketanserin binding

3.3.1 [^{3}H]Ketanserin binding protocol

The procedure for this study was adapted from similar work carried out by Pazos and collaborators (Pazos *et al.*, 1985). Membranes were prepared by pooling the frontal cortex of six male Wistar rats (approx. 1.15 g wet weight). The tissue was then immediately placed in ice-cold 0.32 M sucrose solution (1:10 wt/vol), and homogenized (Ultra Turrax, 1/3 maximum speed for 30 seconds). The homogenate was further homogenized using a teflon mortar and pestle (Tri-R Stir-R, model K 41), four slow up and down strokes at 1/3 maximum speed. The homogenate was then centrifuged (Beckman Ultracentrifuge, model L8-M) at 900 x g for 10 minutes. The supernatant fluid (lacking cell debris) was then centrifuged at 70,000 x g for 15 minutes. The resulting pellet was suspended in ice-cold Tris-HCl buffer (50 mM, p.H. 7.5) at a concentration of 1:10 wt/vol. It was homogenized (Ultra Turrax for 30 sec.) and the suspension incubated at 37°C (Grant, model JB 2) for 15 minutes. The suspension was then centrifuged again at 70,000 x g for 15 minutes. The final pellet was resuspended in buffer (p.H. 7.7), containing 50 mM Tris-HCl, 4 mM $CaCl_2$ and 0.1% ascorbic acid (with 1 µM pargyline being added prior to the binding experiment). When prepared, the membranes were stored in liquid nitrogen until used. They were not kept for more than 4 days. The membrane protein was determined using the assay described by Bradford, 1976.

The preliminary studies performed with [^{3}H]ketanserin were similar to those already described for [^{3}H]5-HT binding, with minor differences. Saturation studies were also performed in exactly the same manner as those described for the binding of [^{3}H]5-HT to membranes of the cerebral cortex (see section 3.1.10). However, binding experiments with [^{3}H]ketanserin were only performed at two time points, corresponding to mid-light and mid-dark of the light : dark cycle. Mianserin hydrochloride (1 µM) was used to define non-specific binding.

3.3.2 Calculations and Statistics

The results of saturation studies were analysed as described in section 3.1.13.

Student's t-test was used for the statistical comparison of the results obtained at mid-light and mid-dark, of the light : dark cycle.

3.3.3 Drugs

[^{3}H]ketanserin 64.9 Ci/mmol (NEN research products, was a gift from Beecham pharmaceuticals, Harlow, Essex, England), mianserin hydrochloride (Beecham pharmaceuticals, Epsom, Surrey, England), pargyline hydrochloride (Sigma) and polyethyleneimine (Sigma).

3.4 RESULTS OF [^{3}H]Ketanserin binding studies

The [^{3}H]ketanserin used in this study was 97.8% pure; all experiments were completed within three months of this analysis.

3.4.1 Investigation of the serial dilution of [^{3}H]Ketanserin concentrations

[^{3}H]ketanserin was obtained from NEN (Du Pont), dissolved in an ethanolic solution. This solution posed a considerable problem in the preparation of standard ligand concentrations for use in saturation experiments. When serial dilutions were prepared using Tris-HCl buffer, the counts produced by some of the concentrations were suggestive of up to a 30% loss of ligand. The problem was thought to be attributable to either the binding of the ligand to pipette tips and tubes used in the preparation of serial dilutions or the inability of the ligand to dissolve in buffer. Several different makes and compositions of tubes were tried, however the problem was still evident. In addition, aging of the tubes had little effect on the loss. Also, when serial dilutions were prepared in a closed system (no-loss) manner, a considerable amount of ligand was still being lost, suggesting that it was binding to pipette tips, since this was the only variable. Serial dilutions prepared in distilled water were still associated with a loss of ligand. However, when dilutions were prepared using 95% ethanol, no loss in counts was observed. Overall, the problem was probably a mixture of the ligand binding to pipette tips and tubes as well as its inability to dissolve freely in buffer.

This problem was finally overcome when buffer containing ethanol was used in the

preparation of serial dilutions. A 10% ethanol concentration in Tris-HCl buffer still resulted in more than 5% loss of ligand. However, when a solution of buffer containing 20% ethanol was used, the counts from the respective concentrations were indicative of a less than 2% loss, this value was considered acceptable. It is rather surprising that other authors of binding studies have not encountered similar problems with the ethanolic solution of this ligand. Leysen *et al.* (1982), are the only authors to report that such a problem actually exists and take appropriate action to counter it.

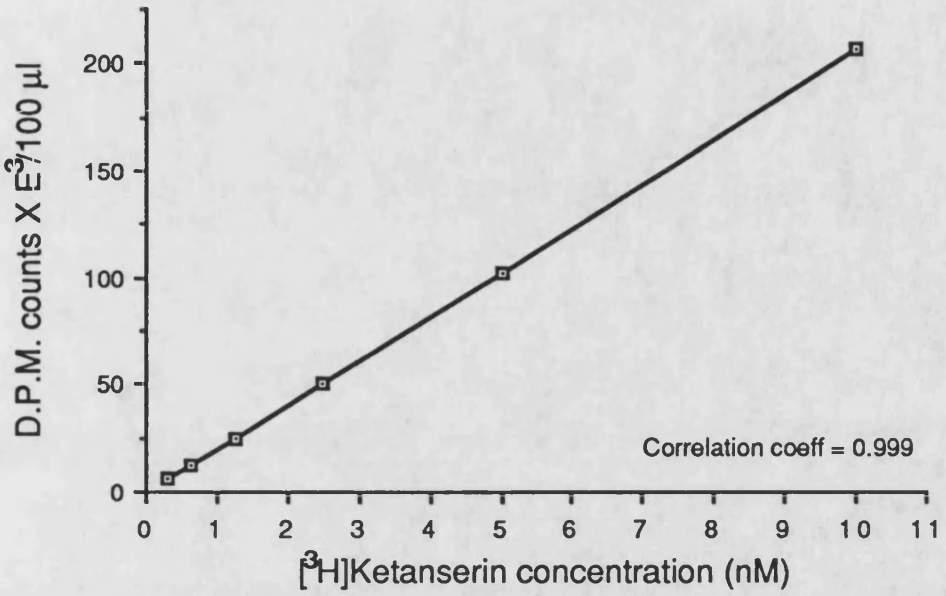
Six different concentrations (0.3125, 0.625, 1.25, 2.5, 5 and 10 nM) of [^{3}H]ketanserin were prepared by serial dilution in polystyrene tubes using ice-cold Tris-HCl (containing 20% ethanol) of the composition used to suspend the final pellet. These concentrations were ten fold higher for reasons described in the serial dilution of [^{3}H]5-HT (see section 3.2.1). A 100 µl solution of each of these concentrations was counted in duplicate. A good correlation was seen when D.P.M. counts were plotted against [^{3}H]ketanserin concentration (Fig. 31), suggesting that pipetting was satisfactory and that [^{3}Hketanserin dissolution was uniform.

The standard concentrations of ligand were prepared in Tris-HCl buffer containing 20% ethanol, a ten fold dilution occurred during membrane binding studies, which meant that the ethanol was present only at a concentration of 2%. This concentration is considered unlikely to interfere with the binding (Lunt, personal communication).

3.4.2 Extent of [^{3}H]Ketanserin binding to filter paper

Filter papers were either kept dry or soaked for 90 minutes or Tris-HCl buffer or 0.1% polyethyleneimine. Following this soaking period, 1 ml of [^{3}H]ketanserin (about 3 nM) containing no membranes was filtered, and the filters washed twice with 5 ml Tris-HCl buffer. The amount of radioactivity binding was lowest in filters that had previously been soaked in 0.1% polyethyleneimine (Table 14). As a result, filters were routinely soaked in a solution of 0.1% polyethyleneimine prior to use.

Figure 31. Effect of the serial dilution of [^{3}H]Ketanserin against D.P.M. counts



The serial dilutions were performed in polystyrene tubes using Tris-HCl containing 20% ethanol. The results are the means±s.e.m of two separate experiments each performed in duplicate.

3.4.3 Investigation of washing

Rat frontal cortex membranes (0.3 mg protein) were incubated at 37°C with [^{3}H]ketanserin (0.2 nM) for 30 minutes, in the presence or the absence of 1 μM mianserin to determine non-specific and total binding respectively. The membranes were then filtered on GF/B filters and washed either two, three or four times with 5 ml ice-cold Tris-HCl buffer. There was little difference between the counts remaining after washing either two, three or four times with 5 ml buffer (Table 15). As a result, a washing procedure involving two 5 ml washes with ice-cold buffer was chosen for subsequent studies.

3.4.4 Specific binding of [^{3}H]Ketanserin at varying protein concentrations

Increasing protein concentration in the incubation was associated with a linear increase in the total and the specific binding components, up to about 0.4 mg protein (Fig. 32). In contrast, non-specific binding increased linearly with protein concentration. From this study, a membrane protein concentration of 0.3 mg was chosen for future work on the grounds that it falls within the linear range of the specific binding line.

3.4.5 Investigation of protein loss through filters

No protein was detected when the filters were washed twice with 5 ml Tris-HCl buffer. As a further check, the membranes were washed with just 2 ml of ice-cold Tris-HCl buffer. It was found that approximately 18.7% (n=2) of the protein used (0.3 mg per incubation), was recovered in the filtrate. This loss is more likely to reflect the filtering of cellular components rather than membranes, resulting from the less stringent washing procedure employed in the preparation of membranes. It is nevertheless a source of error.

3.4.6 Specificity of [^{3}H]Ketanserin binding to membranes

The replacement of membranes with Tris-HCl buffer was associated with a marked reduction in the total and non-specific binding of [^{3}H]ketanserin (Table 16), suggesting that the ligand is binding to membranes. In addition, denaturation of membrane proteins by

Table 14. Effects of various treatments on the binding of [^{3}H]Ketanserin to filter paper

Treatment	D.P.M. counts (mean±s.e.m.)
soaked in Tris-HCl	2613±318
soaked in 0.1% polyethyleneimine	1557±104
kept dry	2177±207

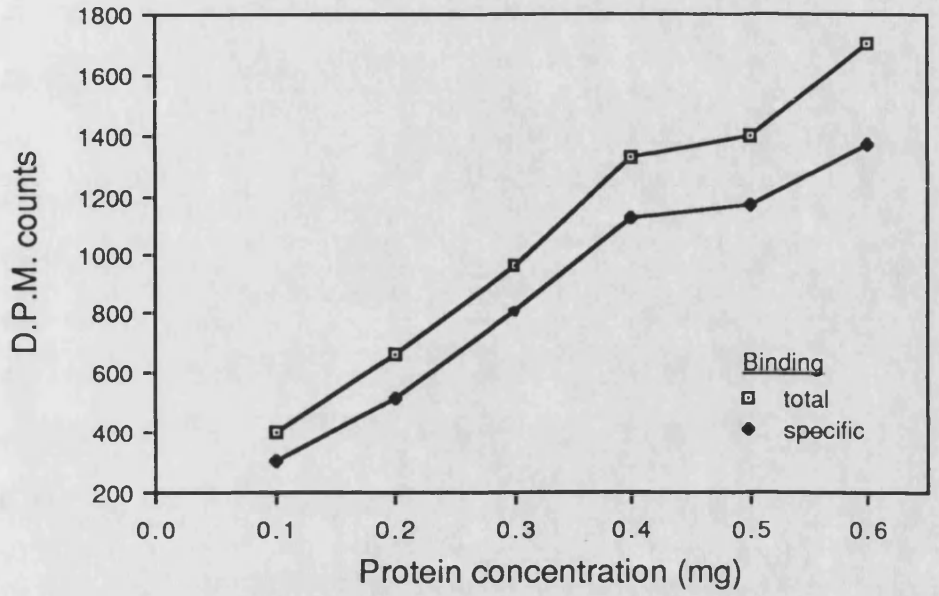
The filter papers were soaked in the appropriate solution for at least least 90 minutes, after which 1 ml of about 3 nM [^{3}H]ketanserin was filtered, followed by two 5 ml washes with ice-cold Tris-HCl buffer. The counts represent the means ± s.e.m. (n=4) of [^{3}H]ketanserin bound to the filters.

Table 15. Effect of the number of washes on the total binding of [^{3}H]Ketanserin

volume	number of washes	time taken (seconds)	C.P.M. counts (specific binding)
5 ml	2	12	475
5 ml	3	17	482
5 ml	4	21	491

Membranes (0.3 mg protein) of rat frontal cortex were incubated at 37°C with [^{3}H]ketanserin at a concentration of 0.2 nM, for 30 min. The membranes were then filtered and washed either two, three or four times with 5 ml of ice-cold Tris-HCl buffer. Specific binding was calculated by subtracting non-specific binding (using 1 µM mianserin) from total binding. The counts are the means from a single experiment performed in duplicate.

Figure 32. Effect of varying protein concentrations on the total and specific binding of [^{3}H]Ketanserin



The results are presented as mean C.P.M. counts of a single experiment performed in duplicate. Total binding was measured using 0.2 nM [^{3}H]ketanserin and non-specific binding was that present after the addition of 1 µM mianserin. Specific binding was calculated by subtracting non-specific binding from the total binding.

boiling resulted in the complete loss of specific [^{3}H]ketanserin binding. These results add further support to the idea that [^{3}H]ketanserin specifically binds to a protein component of membranes.

3.4.7 [^{3}H]Ketanserin binding to membranes of the rat frontal cortex at mid-dark and at mid-light

Saturation studies were performed on membranes (0.3 mg protein per incubation) prepared at mid-dark and at mid-light using six different concentrations of [^{3}H]ketanserin (from 0.02 to about 1 nM). The actual concentration of ligand present in the incubation was estimated from the counts produced by a 100 µl sample of the standard, counted in duplicate. Non-specific binding was defined as that measured in the presence of 1 µM mianserin. Non-specific [^{3}H]ketanserin binding increased linearly with increasing [^{3}H]ketanserin concentration (Fig. 33), confirming that the washing procedure removed all the washable non-specifically bound ligand. A similar linear relationship of non-specific binding was seen in experiments performed at mid-dark.

The specific binding of [^{3}H]ketanserin obtained by subtracting non-specific from the total binding represented over 80% of total binding. It increased gradually and was starting to plateau at the highest concentration of ligand used (Fig. 33). Similar curves were produced by experiments performed at the mid-dark time point. A Scatchard plot of this data produces a straight line (Fig. 34), suggesting that [^{3}H]ketanserin was binding to a single site. The K_D and the B_{max} values were calculated from Scatchard plots for each experiments by regression analysis. No significant difference in the kinetic properties of [^{3}H]ketanserin binding was found in K_D and B_{max} values obtained at mid-dark were compared to those obtained at mid-light (Table 17). These results suggest that neither the affinity nor the density of the 5-HT_2 binding sites as labelled by this ligand is altered, at these time points.

In the incubation medium, ethanol was present at a concentration of 2%. This may have contributed to the slightly higher K_D values and also the inability to fully reach saturation. In addition, the K_D and B_{max} values can be reduced by the use of electrolytes, ascorbic acid and pargyline (Leysen *et al.*, 1982).

Table 16. Specificity of [^{3}H]Ketanserin binding to membranes of rat frontal cortex

	0.8 ml membranes 0.1 ml [^{3}H]ketanserin	0.8 ml Tris-HCl 0.1 ml [^{3}H]ketanserin
0.1 ml mianserin :	729	370
0.1 ml Tris-HCl :	6008	527

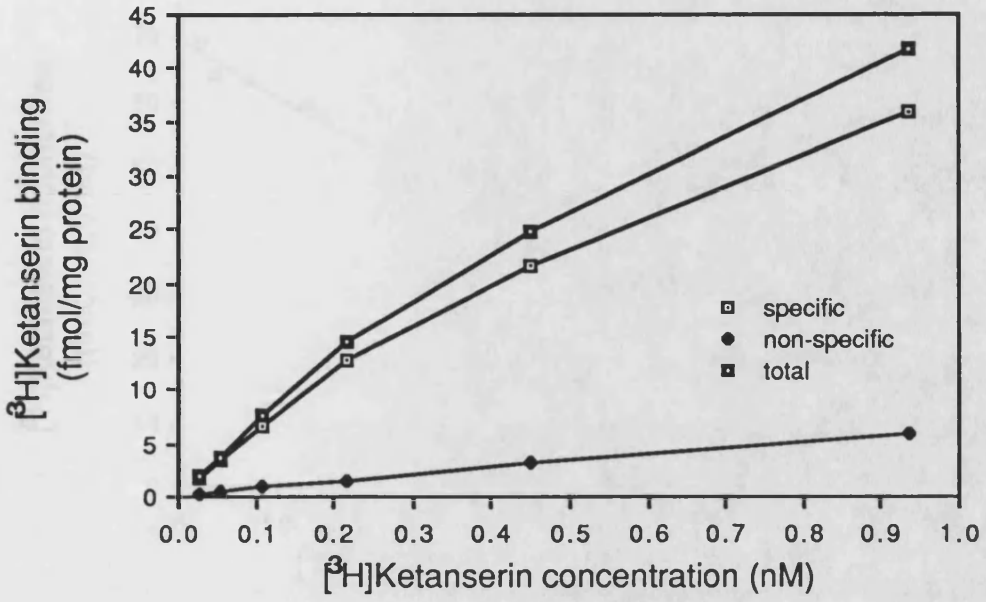
Results are the mean D.P.M. counts of a single experiment performed in triplicate. Incubations were performed in a number of combinations using membranes (0.3 mg protein in 0.8 ml), 0.1 ml [^{3}H]ketanserin (0.23 nM final conc.), 0.1 ml Tris-HCl and mianserin (1 µM final conc.)), for 30 minutes and then filtered on GF/B filters. The results are expressed as the counts remaining following two washes with 5 ml ice-cold Tris-HCl buffer.

Table 17. Estimation of the affinity constant and the number of [^{3}H]Ketanserin binding sites in membranes of rat frontal cortex

	mid-dark	mid-light
K_D (nM) :	1.1916±0.078	1.1975±0.120
B_{max} (fmol/mg protein) :	61.14±6.48	63.69±7.62

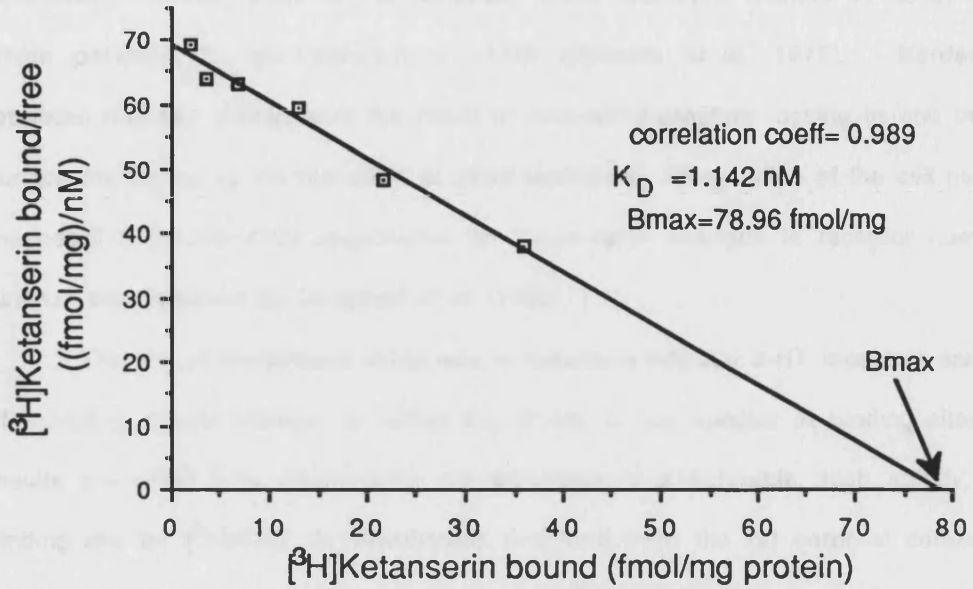
Specific 5-HT_2 binding was obtained by calculating the difference between total [^{3}H]ketanserin binding and that measured in the presence of 1 µM mianserin. Scatchard plots of this data were analysed by linear regression. The affinity constant (K_D) and the number of [^{3}H]ketanserin binding sites (B_{max}) were determined for each experiment, at mid-dark and at mid-light. Each value represents the mean ± s.e.m. of four separate experiments, each performed in duplicate.

Figure 33. Variation of [^{3}H]Ketanserin binding with concentration



Increasing concentrations of [^{3}H]ketanserin (0.025 - 0.937 nM) were incubated with membranes (rat frontal cortex at mid-light) in the absence and in the presence of 1 µM mianserin (non-specific binding). Specific binding was calculated by subtracting the non-specific binding from the total binding, expressed as fmol/mg protein. Each point represents the mean of a single experiment performed in duplicate.

Figure 34. Scatchard plot of the specific binding of [^{3}H]Ketanserin to membranes of rat frontal cortex



Scatchard plot of the saturation data of specific [^{3}H]ketanserin binding. Specific binding was calculated by subtracting the non-specific binding from the total binding, expressed as fmol/mg protein. This data is from a single assay (rat frontal cortex at mid-light) and is representative of four separate experiments performed at this time point. Each point represents the mean of a duplicate determination.

3.5 DISCUSSION

Circadian rhythms have been reported in a number of neurotransmitter receptor binding sites, in several regions of the rat brain (Kafka *et al.*, 1986). In addition, *beta*-adrenoceptor binding sites in the rat pineal gland display a marked circadian variation, which parallels the accumulation of cAMP (Romero *et al.*, 1975). Harden (1983) proposed that this change was the result of *beta*-adrenoceptors moving in and out of the surface membrane by the formation of small vesicles by invagination of the cell membrane; the possible mechanism/s responsible for these rapid changes in receptor number and function are discussed by Campbell *et al.* (1985).

The aim of the present study was to determine whether 5-HT receptors are capable of exhibiting similar changes in either the affinity or the number of binding sites. The results presented here demonstrate the existence of a saturable, high affinity, specific binding site for [^{3}H]5-HT, in membranes prepared from the rat cerebral cortex. The affinity of binding and the maximum number of [^{3}H]5-HT binding sites agree with those of Herrick-Davis and Titeler (1988), who also reported the existence of a high affinity [^{3}H]5-HT binding site in this region, with a K_D of 2.1 nM and a B_{max} of 245 fmol/mg protein. This [^{3}H]5-HT binding site has been identified in a number of regions of rat brain (Pazos and Palacios, 1985 ; Herrick-Davis and Titeler, 1988), as well as in the brains of a number of other species (Schnellmann *et al.*, 1984 ; Heuring and Peroutka, 1987).

The pharmacological characterization of this [^{3}H]5-HT binding site was not considered necessary because the results of kinetic, saturation and competition experiments suggest that low nanomolar concentrations of this ligand bind to a single class of 5-HT binding sites, designated 5-HT$_1$ (Peroutka and Snyder, 1979 ; Nelson, 1988).

The affinity of [^{3}H]5-HT binding to the 5-HT$_1$ binding site in the membranes of rat cerebral cortex, and the maximum number of binding sites, were not significantly different in membranes prepared at the four different time points of the light : dark cycle. Wesemann *et al.* (1983) also reported that the affinity of [^{3}H]5-HT binding during the light : dark cycle was unaltered, but they observed a circadian variation of small amplitude in the number of binding sites when using 5 nM [^{3}H]5-HT and one of greater amplitude when

using 20 nM [^{3}H]5-HT. This rhythm in the binding, at 20 nM [^{3}H]5-HT varied inversely with the concentration of endogenous 5-HT (Wesemann *et al.*, 1986). Their use of such a high concentration of [^{3}H]5-HT (20 nM) suggests that binding took place to a site which is different from the high affinity 5-HT$_1$ binding site. High and low affinity binding sites for [^{3}H]5-HT have been observed by a number of authors (for an extensive list the reader is referred to Nelson, 1988); however, it is at present a matter of controversy whether these binding sites represent physically distinct molecular entities or whether they represent different states of the same binding site (Nelson, 1988). Furthermore, the use of crude brain homogenates by Wesemann *et al.* (1986) is likely to have yielded results which reflect the summation of rhythms from a number of brain regions. In addition, the observed circadian rhythm in 5-HT concentrations would, if membranes were not washed properly, produce an inverse rhythm in [^{3}H]5-HT binding. These particular obstacles were overcome in the present study by incubating membranes for 15 minutes at 37°C, in order to enable the endogenous 5-HT to be metabolized by MAO. Also, the tissue was osmotically shocked, twice (to burst nerve endings and disrupt vesicles containing endogenous neurotransmitter), and the thorough membrane washing procedure ensured that the circadian variation in 5-HT concentrations (Quay, 1968) would not interfere with the binding of [^{3}H]5-HT.

As the 5-HT$_1$ binding site is heterogeneous (Pedigo *et al.*, 1981 ; Herrick-Davis and Titeler, 1988), it is possible that the individual rhythms of its component subsites could summate and cancel out. For this reason, competition studies are useful for the subclassification and quantification of a single heterogeneous binding site into its individual components. To date, the 5-HT$_1$ binding site is reported to be composed of five separate binding subtypes (See section 1.3.2.1). The potent 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, when present at a concentration of 100 nM, prevents the binding of [^{3}H]5-HT to the 5-HT$_{1A}$ subtype (Heuring and Peroutka, 1987), and also selectively displaces [^{3}H]5-HT from the 5-HT$_{1A}$ subtype (Middlemiss and Fozard, 1983). These 5-HT$_{1A}$ binding sites in the cortex probably correspond to the post-synaptic 5-HT$_{1A}$ receptor (Verge *et al.*, 1986 ; Crino *et al.*, 1990). In contrast, the 5-HT$_{1B}$ binding sites in the cortex are located located pre- and post-synaptically (Crino *et al.*, 1990).

Pindolol, at a concentration of 1 μM, selectively displaces (or prevents) the binding of [^{3}H]5-HT to the 5-HT$_{1A}$ and the 5-HT$_{1B}$ binding sites (Herrick-Davis and Titeler, 1988). Propranolol, like pindolol, is also a *beta*-adrenoceptor antagonist, with efficacy for preventing or displacing [^{3}H]5-HT binding from the 5-HT$_1$ binding site (Nahorski and Willcocks, 1983). It is, however, not as selective as pindolol, and displaces [^{3}H]5-HT binding from the 5-HT$_{1A}$, 5-HT$_{1B}$, and the 5-HT$_{1C}$ binding subtypes, and only weakly from the 5-HT$_{1D}$ subtype (Hoyer *et al.*, 1987). The ability of propranolol to displace the binding of [^{3}H]5-HT from the 5-HT$_{1E}$ binding site has not been investigated.

In the present study, 8-OH-DPAT (100 nM), produced a reduction in the binding of [^{3}H]5-HT to rat cortical membranes, the size of which was not significantly different at the four time points. This 5-HT$_{1A}$ component accounted for about 50% of the total [^{3}H]5-HT binding taking place, a value which correlates well with that reported by Middlemiss and Fozard (1983) and Herrick-Davis and Titeler (1988). Furthermore, the amount of [^{3}H]5-HT binding still evident in the presence of 8-OH-DPAT (i.e. non-5-HT$_{1A}$) was not significantly different at the four time points.

The presence of propranolol (1 μM) in the incubation medium consistently produced a greater reduction (about 72%) in the binding of [^{3}H]5-HT, when compared to 8-OH-DPAT in membranes of rat cerebral cortex. The magnitude of this reduction was very similar at the four time points, however, these results are difficult to interpret because of the lack of selectivity of propranolol at this concentration. It is likely that propranolol partially prevented the binding of [^{3}H]5-HT to the 5-HT$_{1A}$, 5-HT$_{1B}$ and the 5-HT$_{1C}$ subtypes. With hindsight, the choice of this agent for use in competition studies was not an appropriate one. As a consequence, the results obtained with the use of propranolol could only be interpreted qualitatively. Moreover, if a drug had been used successfully to prevent [^{3}H]5-HT binding to the 5-HT$_{1B}$ binding site, the results would still have been difficult to interpret because these sites are located both pre- and post-synaptically (Crino *et al.*, 1990). As a consequence, it is not possible to correlate the results of binding studies with those measuring the functional activity of the terminal 5-HT autoreceptor.

The results of competition studies performed on membranes of the hamster

cerebral cortex were similar to those found for the rat, suggesting that these two species contain a similar complement of 5-HT binding sites. However, in cortical membranes, prepared from the guinea-pig brain, propranolol (1 μM) produced a smaller reduction (about 33%) in the binding of [^{3}H]5-HT, in comparison to the 50% produced by 8-OH-DPAT. This difference is likely to result from the partial ability of propranolol to prevent the binding of [^{3}H]5-HT to the 5-HT$_{1A}$ subtype (Hoyer *et al.*, 1987), and may also be related to the absence of the 5-HT$_{1B}$ binding site in the guinea-pig (Heuring *et al.*, 1986). In place of the 5-HT$_{1B}$ binding site, the guinea-pig is reported to possess the 5-HT$_{1D}$ binding site, for which propranolol has a lower affinity (Hoyer and Middlemiss, 1989).

The daily administration of methiothepin (10 mg/kg i.p.) to rats produced little change in the total number of 5-HT$_1$ binding sites or its displaceable components in the cerebral cortex, suggesting that 5-HT$_1$ binding sites are resistant to change following blockade. A similar finding has also been reported by Peroutka and Snyder (1980) and Blackshear *et al.* (1983). In contrast, the same treatment was associated with an up-regulation or an increased sensitivity of the terminal 5-HT autoreceptor, as reported in the superfusion studies section. The inability to detect changes in receptor number is attributed to the lack of specificity of propranolol, and also to the low numbers of presynaptic 5-HT$_{1B}$ binding sites. In addition, a change may have been produced in the affinity of [^{3}H]5-HT binding, but this was not measured.

In the cerebral cortex of the rat, 5-HT concentrations display a circadian variation, values being generally higher during the light phase in comparison to the dark phase (Quay, 1968). In contrast, the *in vivo* release of 5-HT, is greater during the dark phase (Cespuglio *et al.*, 1983), when the firing rate of 5-HT neurones is highest and the animals are awake (McGinty and Harper, 1976). Although the release of 5-HT is greater during the dark phase, the unaltered affinity and number of 5-HT$_1$ binding sites corresponding to receptors in this region, over 24 hours, suggests that these receptors would undergo greater activation during the dark phase. These 5-HT receptors would therefore be expected to pass on any changes applied to them. Changes at the level of the cortex, although easily studied, have little bearing on any of the 5-HT receptor-mediated behaviours so far identified. Clearly, [^{3}H]5-HT or [^{3}H]8-OH-DPAT binding and 5-HT

release need to be investigated at the level of the brainstem or the spinal cord to enable any correlation with the apparent absence of a circadian variation in the 5-HT behavioural syndrome.

The radioligand binding studies using [^{3}H]ketanserin have demonstrated the existence of a saturable, high-affinity, specific binding site in membranes prepared from the rat frontal cortex. The K_D and the B_{max} values for the binding of [^{3}H]ketanserin to this site are similar to those reported by Leysen *et al.* (1982) and Pazos *et al.* (1985). The K_D values observed in the present study were slightly lower than those previously reported (Leysen *et al.*, 1982 ; Pazos *et al.*,1985), and are considered to result from the presence of $CaCl_2$, ascorbic acid and pargyline in the incubation medium (Leysen *et al.*, 1982). In contrast, the presence of these agents is reported to be associated with an underestimation of the B_{max} value (Leysen *et al.*, 1982). Furthermore, an underestimation of the B_{max} value would also be expected because of the protein loss experienced during filtering. As a consequence, the actual "real" B_{max} is likely to be higher than that observed in the present study. It is not known to what extent this binding of [^{3}H]ketanserin reflects the presence of the different states or subtypes of the 5-HT_2 site (see section 1.3.2.2). In addition, a small proportion of [^{3}H]ketanserin binding at high concentrations may reflect its binding to either *alpha*$_1$-adrenergic and/or H_1-histaminergic sites (Leysen *et al.*, 1981 ; Fischette *et al.*, 1987).

Binding studies using [^{3}H]ketanserin were performed on membranes prepared at mid-light and mid-dark, of the light : dark cycle, and did not reveal any significant differences in either the affinity or the total number of binding sites. There is conflicting evidence in the literature for the presence of a circadian variation in the binding to the 5-HT_2 site; for example, Lauro *et al.* (1986) were unable to observe a circadian variation in the binding of [^{3}H]spiperone to the 5-HT_2 binding sites in the cerebral cortex, hypothalamus or the brain stem, similarly Koshikawa *et al.* (1988) were unable to detect a rhythm in the number of cortical 5-HT_2 binding sites, using [^{3}H]ketanserin. In contrast, a marked circadian variation in the binding of [^{3}H]spiperone was reported for the rat forebrain (Bruinink *et al.*, 1983). The reason for this discrepancy is not known.

The failure to observe a significant difference in the binding of [^{3}H]5-HT or [^{3}H]ketanserin to the 5-HT$_1$ and the 5-HT$_2$ binding sites respectively, may be a reflection of the timing of the sampling, or the method of membrane preparation, or the use of the whole cortex rather than specific cortical regions. In addition, it may be that the regions were arrhythmic at the time of the year that the binding was performed, or that rhythms were cancelled by the "pooling" of the brain regions from several animals. Also, rhythms may not be prevalent in different strains of the some species (Quay, 1968). Rhythms can also be obscured if their amplitude of cyclic oscillation is too low. The amplitude of binding rhythms is also influenced by the age of the animal under investigation (Jenni-Eiermann *et al.*, 1985). Furthermore, radio-ligand binding only detects the total number of binding sites and does not distinguish between functional and non-functional receptors, hence the findings of this technique may be useful only as a general indicator of receptor change. The majority of radio-ligand binding studies have investigated the changes in receptor number because changes in this parameter are more likely to result from the exposure or covering of available binding sites, and also because receptor synthesis is a slow process. Affinity changes have not been investigated thoroughly and it is possible that a change in affinity could go undetected and a wrong conclusion be drawn.

Several behaviours attributed to 5-HT receptor stimulation have also been investigated for a circadian component. The head-twitch response (5-HT$_2$ receptor-mediated) is reported to display a marked circadian variation with higher scores at mid-light, whilst the 5-HT behavioural syndrome (5-HT$_1$ receptor-mediated), or its individual components, were devoid of a circadian component (Moser and Redfern, 1985). In addition, other behavioural effects of 5-HT$_1$ receptor stimulation, such as the 8-OH-DPAT-induced hypothermia and the RU 24969-induced hyperlocomotion, are free of any circadian variation (Moser, 1986).

The circadian variation observed in the head-twitch response is unlikely to be secondary to a pharmacokinetic variation. This variation may be the consequence of a change in the number of 5-HT$_2$ receptors or their sensitivity. The circadian variation in the behavioural response to 5-HT$_2$ receptor stimulation parallels the daily variation in neuronal 5-HT concentrations. However, the observed variation in neuronal 5-HT

concentrations is related inversely to the release of 5-HT, which is greater during the dark phase. Therefore, when 5-HT release, and 5-HT neuronal firing, are highest, the 5-HT_2 receptors mediating the head-twitch response are less sensitive to stimulation and *vice versa*. This arrangement would mean that these post-synaptically located 5-HT receptors although less sensitive, are capable of detecting changes in presynaptic neuronal activity. If however, these post-synaptic receptors were most sensitive at the same time as 5-HT release was greatest, then these receptors would be maximally stimulated during the dark phase and unresponsive during the light phase. In this condition, any changes in presynaptic activity are likely to go undetected.

Moser (1986) proposed that presynaptic neuronal activity drives the changes seen in 5-HT_2 receptor activity, causing the activity of these receptors to "up-regulate" and "down-regulate" when presynaptic activity is lowest and highest respectively. Support for this theory comes from ontogenetical studies, where the rhythm in 5-HT concentrations only develops fully 35 days after birth (Okada, 1971), whereas the rhythm in 5-HT_2 binding sites is established not at 30 days but at 90 days (Bruinink *et al.*, 1983). The time course of development of a rhythm in the release of 5-HT has not been investigated, but its findings would be of great interest.

In a recent study, the administration of phenelzine followed by either citalopram or paroxetine was associated with an unaltered K_D but a reduction in the number of 5-HT_2 binding sites in the cerebral cortex, and also a reduction in the number of wet-dog shakes, three hours after drug administration (Koshikawa *et al.*, 1985). These drugs probably caused an increase in the synaptic concentration of 5-HT, leading to the increased activation of the 5-HT_2 receptor, and its consequent down-regulation. However, these authors failed to mention whether this study was performed during the light or the dark phase. Rapid changes in either the functional activity or the number of 5-HT_1 binding sites have not been investigated.

The head-twitch response is considered to be mediated *via* the activation of 5-HT_2 receptors located in the brainstem (Bedard and Pycock, 1977). A correlation of the number and affinity of the 5-HT_2 binding sites in this region, over 24 hours, would be of fundamental importance; however, the majority of binding studies have looked for changes

in the cerebral cortex, where the greatest density of 5-HT_2 binding sites are found (Leysen *et al.*, 1982). Lauro *et al.* (1986) are the only authors to have investigated the circadian variation in the binding to 5-HT_2 sites in the brainstem. They reported the number of 5-HT_2 binding sites to be unchanged over 24 hours, but these authors made no attempt to determine the affinity of binding over 24 hours.

An alternative hypothesis is that the circadian rhythm in the head-twitch response is not due to changes at the level of the 5-HT_2 receptor, but the consequence of a change(s) further down-stream of this receptor (*i.e.* a variation in the secondary messenger system or in the neuronal pathway/s linking this receptor to the initiation of the head-twitch response).

With reference to the 5-HT behavioural syndrome, neither the total syndrome or its individual components display a circadian variation. Indeed, the behaviours attributed to 5-HT_1 receptor stimulation would be more likely to display a variation in response because of the greater affinity of 5-HT for the 5-HT_1 binding site, and also because of the circadian variation in the release of 5-HT. However, the circadian rhythm in the release of 5-HT in the brainstem has not been investigated. The lack of a rhythm in 5-HT_1 receptor mediated behaviours is probably the result of a damping effect caused by compensatory changes.

Clearly, more fundamental research needs to be performed in order to determine the cause and the consequence of the circadian variation in the head-twitch response and also the apparent lack of variation of the behaviours attributed to 5-HT_1 receptor stimulation.

4 SOMADENDRITIC 5-HT AUTORECEPTOR SENSITIVITY ESTIMATION

4.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

The indole ring of L-tryptophan and its metabolic derivatives, is readily prone to oxidation with the subsequent release of electrons. The electrons released can be measured as an electric current, the size of which is proportional to the amount of compound oxidized. The basis of electrochemical detection is that oxidation is made to take place at the surface of a carbon (graphite) electrode which acts as the oxidizing agent.

The presence of certain oxidizable groups (*e.g.* -OH or -NH) determine the potential at which oxidation takes place. 5-HTP, 5-HT and 5-HIAA, all of which are hydroxylated on the indole ring, oxidize at lower potentials (about + 0.50 V), while a higher potential (about + 0.90 V) is required to release the electron from the nitrogen in the indole ring of L-tryptophan.

The majority of HPLC columns used with electrochemical detectors are of the reverse phase type. Reverse phase systems consist of a non-polar stationary phase and a polar mobile phase. The stationary phase is usually composed of fine silica particles (5-10 μM diameter), on to which are bonded hydrocarbon alkyl chains, commonly octadecylsilyl (ODS, C_{-18} columns). The mobile phase is usually a mixture of buffer and methanol. Under such a system, polar compounds are poorly retained on the column and are consequently eluted first. An ion-pairing agent is sometimes added to the mobile phase. This is usually a bulky group which ion pairs with a compound of the opposite charge and increases its hydrophobic character, thus retarding its rate of passage down the column and aiding separation. The ion-pairing agent used in this study was sodium octane sulphonic acid.

4.2 METHODS

4.2.1 The HPLC system

The column (length 15cm and diameter 6mm) was packed with Hypersil ODS (5 μm particle size, Shandon) in the department. It was connected to the rest of the chromatographic system which consisted of the following : LKB (bromma) 2150 HPLC pump ; amperometric electrochemical detector with working glassy carbon and reference

Ag/AgCl electrodes (Bioanalytical Systems Inc., model LC-4A) ; Rheodyne injection valve (model 7125) fitted with a 100µl injection loop (Fig. 35). Samples were injected into the loop using a 100µl Hamilton HPLC syringe (model 710) and peaks were recorded on a JJ instruments chart recorder (model CR 650 S). The column and the injection loop were immersed in a water bath maintained at a temperature of 29-31°C by a water circulator pump (Grant, model SU 5) and monitored using a temperature probe (RS, 612-849).

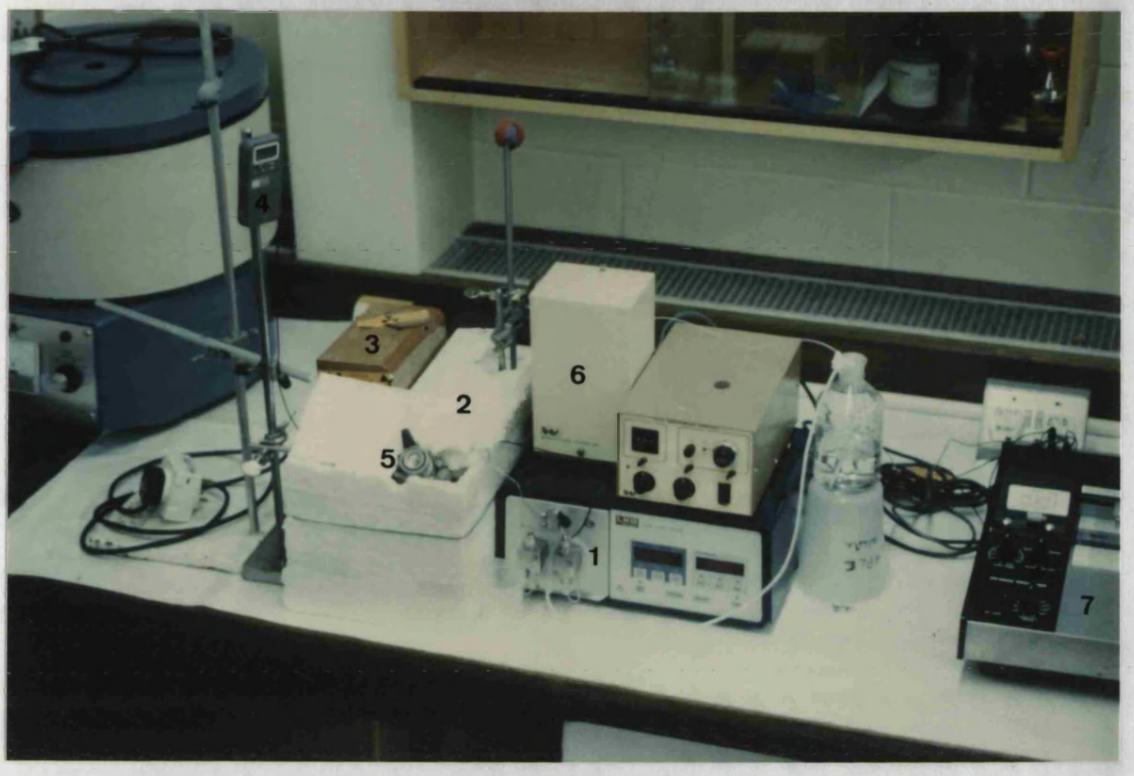
Fresh mobile phase was made up each day using doubly "polished", deionized water prepared using Milli Q water systems (Millipore Corporation). The mobile phase contained : 0.07 M sodium dihydrogen phosphate ($NaH_2 PO_4 . 2H_2O$), 0.3 mM sodium octane sulphonic acid, 0.1 mM EDTA and 8% methanol. The p.H. was adjusted to 2.75 with 6 M perchloric acid. It was vacuum-filtered through 0.2 µm membrane filters (Whatman) and degassed by bubbling helium gas through for 20 min. It was then sonicated for 5 min to remove any bubbles of helium. Mobile phase was pumped at a flow rate of 1 ml/min, and a pressure of 130-150 bar. The sensitivity of the detector was set at 5 nA for full scale deflection. Fresh mobile phase was pumped through the detector each morning for 30 min and then it was left pumping at a reduced flow rate during the day. This procedure resulted in a stable baseline being obtained in the evening. Because of electrical interference a stable baseline was unattainable during the daytime, even after considerable damping had been employed. As a result all samples were injected at night when the baseline was markedly better.

The electrode potential, methanol concentration and p.H. were adjusted to ensure that the 5-HTP peak was sharp and eluted well clear of any interfering peaks. This is described in the method that follows.

4.2.2 The routine maintenance of the HPLC

Every month after disconnecting the column, the HPLC pump was cleaned by pumping warm 30% decomatic detergent (Decon Laboratories) at 5 ml/min for 30 min, followed by several combinations of water/methanol. At the end of each working week, the top of the column was inspected for the presence of a dark deposit. This deposit was scraped off and replaced with a freshly prepared slurry of hypersil ODS in methanol. The

Figure 35. The High Performance Liquid Chromatography System



The HPLC system consisted of: (1) LKB (bromma) HPLC pump; (2) a reverse phase HPLC column located in a insulated water bath; (3) water circulator; (4) temperature probe; (5) rheodyne injection valve fitted with a 100µl injection loop; (6) electrochemical detector with working glassy carbon and reference Ag/AgCl electrodes; (7) JJ chart recorder.

pre-column filter was also replaced at this time. Occasionally, the surface of the glassy carbon electrode was cleaned using alumina polish on a polishing pad. The potential of the reference electrode was checked prior to the experiment. Any air bubbles trapped in its housing were also dislodged.

This routine maintenance of the HPLC system ensured its smooth and relative trouble-free running.

4.2.3 Effect of varying electrode potential on the peak height of 5-HTP

As the electrode potential is increased, then potentially more compounds will be detected, therefore the lowest potential at which the compound of interest is detectable is usually preferred. Since 5-HTP is a hydroxylated indoleamine, it would be expected to oxidize at a potential of + 0.50 V. In order to find the optimum potential for its detection, the peak produced by a standard concentration of 500 pg/100 µl loop, was investigated against varying electrode potentials (Table 18).

Table 18.

Effect of varying oxidation potential on the peak height of the 5-HTP peak

potential (V)	5-HTP peak Height (mm)	baseline noise (mm)
+ 0.50	13	3
+ 0.60	49	1
+ 0.70	50	2
+ 0.80	46	4

On increasing the potential from + 0.50 V to + 0.60 V there was a large increase in the peak height. However, any further increases in the electrode potential did not result in an accompanying increase in the peak height. In general, as the electrode potential is raised, baseline noise is also seen to grow.

It is evident that an electrode potential of + 0.60 V offers not only optimum peak height but also a low background noise. Consequently this electrode potential was used throughout the rest of this study.

4.2.4 Effect of methanol concentration, p.H. and column temperature on the retention time of the 5-HTP peak

Methanol is a commonly used solvent in HPLC systems because it does not interfere with the common methods of detection nor does it react chemically with the type of compounds under investigation. The inclusion of methanol in the mobile phase has the effect of reducing the retention times of all compounds irrespective of their molecular state. It is able to achieve this because of its non-polar nature. As a result it exhibits a very high affinity for the stationary phase, while that of polar compounds is reduced causing them to elute earlier.

Adjustment of the p.H. has the effect of determining the extent to which the compound of interest is ionized/unionized. The ionized/unionized nature of the compound of interest primarily determines whether ion-pairing takes place and this in turn determines its affinity for the stationary phase. However, all columns have a safe working p.H. range depending on their composition and harm may be caused to the column if this range is exceeded.

Manipulation of the column temperature is often used to decrease the retention time of compounds as well as the operating pressure of the pump. Using a fixed column temperature has the advantage of cutting down on laboratory temperature fluctuations (*e.g.* day/night), which would otherwise cause the retention time to be changed. It can also act as an aid to resolution. The results are shown in Tables 19 and 20.

Unfortunately, the initial compositions of mobile phase were investigated with little control over column temperature. However, when it was realized that samples would have to be run at night as a result of excessive electrical interference during the day, column temperature was taken into consideration (Table 20). The general findings of this preliminary work are unlikely to be affected.

Increasing methanol concentration, p.H. and column temperature all had the predicted effect of reducing the retention time. Since these three factors can alter the retention time, their manipulation to different extent allows for the peak of interest to be eluted well clear of any interfering peaks.

Various concentrations of mobile phase were tried with preliminary brain samples.

Table 19.

Effect of varying methanol concentration and p.H. on the retention time for the 5-HTP peak

methanol conc. (%) of buffer	p.H.	retention time of 5-HTP (min/sec)
6	4.0	7.45
6	4.5	6.00
6	5.0	6.00
8	4.0	7.00
8	4.5	6.30
8	5.0	5.45
10	4.0	5.40
12	4.0	4.45
12	4.5	4.25
12	5.0	4.00

In these experiments the column was maintained at room temperature.

Table 20.

Effect of varying methanol concentration, p.H. and column temperature on the retention time for the 5-HTP peak

methanol Conc. (%) of buffer	p.H.	Temp (°C)	retention time of 5-HTP (min / sec)
10	3.0	37	8.00
10	2.5	30	19.20
10	2.5	37	13.20
10	2.75	25	19.00
10	2.75	30	14.40
10	2.75	37	10.40
8	2.75	30	19.15

A mobile phase containing 8% methanol at a p.H. of 2.75 was finally chosen, with the column being maintained at 30°C by means of a water bath. This combination ensured that the 5-HTP peak was sharp and eluted well clear of any interfering peaks.

4.2.5 Estimation of the linearity of peak height to 5-HTP and 5-HIAA concentrations

The relationship of peak height to a variety of standard concentrations of 5-HTP and 5-HIAA was investigated Fig. 36 and 37. A range of standard concentrations was chosen which would cover that expected from treated brain samples.

4.2.6 Effect of 8-OH-DPAT on 5-HTP synthesis in the cerebral cortex examined at mid-light and at mid-dark

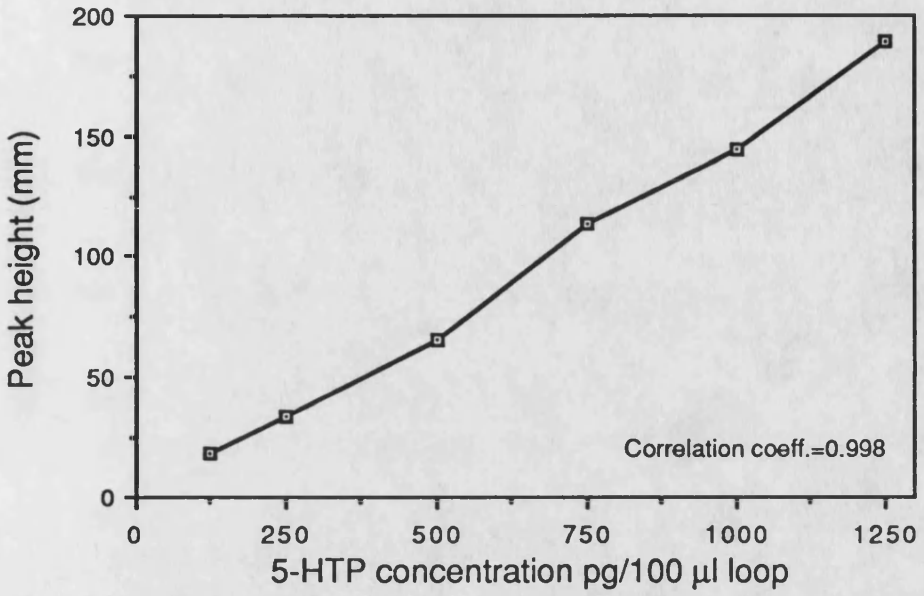
Male Wistar rats (University of Bath strain), were housed in specially constructed wooden cabinets, under controlled conditions for at least 2 weeks prior to use (see section 2.1.1). Some animals were phase shifted to enable all experimental procedures to be carried out during normal working hours. When used in experiments the animals weighed between 190-320g.

8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) was dissolved in 0.9% saline, but required gentle warming and stirring to achieve dissolution. m-hydroxybenzylhydrazine dihydrochloride (NSD 1015), was dissolved in saline, the p.H. being adjusted to 7 with NaOH.

Rats were injected with 8-OH-DPAT (0.01-0.30 mg/kg) or saline subcutaneously and after 30 min again with NSD 1015 (100 mg/kg i.p.). After another 30 min the rats were killed by cervical dislocation. See experimental protocol diagram below :

Figure 36.

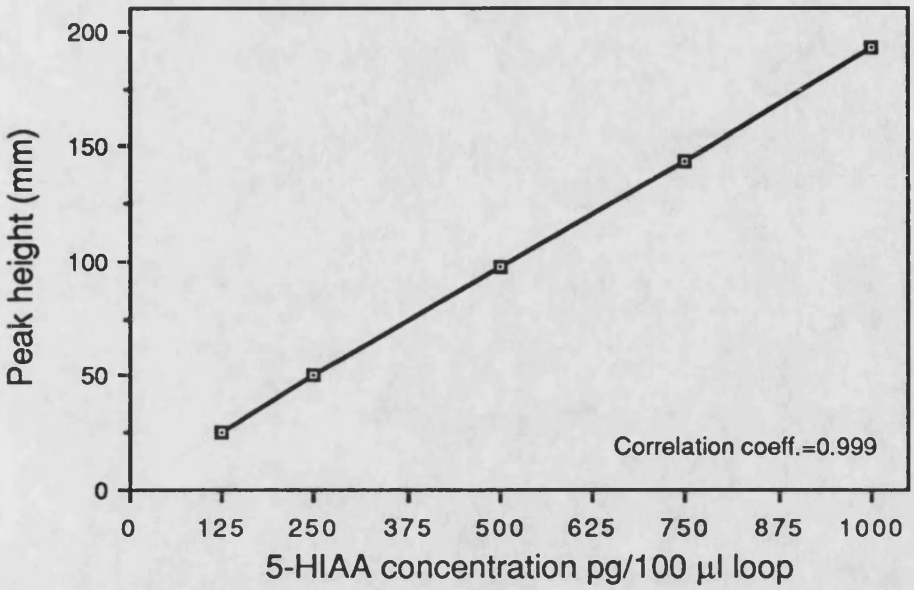
Effect of increasing concentrations of 5-HTP concentrations on the peak height



A range of standard concentrations of 5-HTP were prepared. A 100 µl volume of these solutions was injected into the column (5-HTP t_R=19.15 min/sec), and the peak height measured. The graphs represent the means of two separate experiments.

Figure 37.

Effect of increasing concentrations of 5-HIAA concentrations on the peak height



A range of standard concentrations of 5-HIAA were prepared. A 100 µl volume of these solutions was injected into the column (5-HIAA t_R=23.20 min/sec), and the peak height measured. The graphs represent the means of two separate experiments.

Figure 38.

Procedure for somadendritic 5-HT autoreceptor activity measurement

8-OH-DPAT	NSD 1015	Animal
or saline		killed

I____30_min_____I____30_min_____I

Following decapitation, the brains were rapidly taken out and the cerebral cortex dissected on an ice-cooled petri dish and assayed for their content of 5-HTP.

In addition, a group of rats which received no pharmacological treatment was used to establish basal concentrations of 5-HTP, at mid-light and at mid-dark.

4.2.7 Preparation of samples

All animals were first killed by cervical dislocation in either the light or the dark phase as appropriate and were then subsequently decapitated. Their brains were then rapidly removed and placed on an ice-cooled petri dish. The cerebral cortex was then dissected and weighed. It was homogenized using a teflon mortar and pestle (Tri-R instruments), at a concentration of 10% w/v using 0.1 M perchloric acid (p.H. 1.47, at 4°C). This 0.1 M solution of perchloric acid was prepared using Milli Q water. The homogenate was then transferred to centrifuge tubes (Ultra-clear, Beckmann), shaken for 1 min using a whirlimixer (Fisons) and centrifuged at 4°C and 15,000 x g for 15 min using a Beckmann ultracentrifuge (model L 8-M and a Ti 70.1 rotor). The clear supernatant was filtered by injecting through a 0.45 μm pore size filters (Acro LC 13, Gelman sciences). It was then immediately frozen at -19°C. Samples were kept frozen for no longer than 16 days. The results obtained with aged aqueous standards suggested that 5-HTP underwent little degradation over several weeks, if stored at 4°C. Prior to injection each sample was defrosted to room temperature and whirlimixed. Three injections of 100μl each were made using a Hamilton syringe to first clean the loop, the fourth 100μl injection was then allowed to warm up in the loop prior to injection onto the column.

An aqueous standard was injected after every third brain sample to check that the efficiency of the column had not changed. Sometimes, the standard was injected twice consecutively, this was only done if the peak produced by the first injection was ± 5% different from the pre-sample value.

4.2.8 Efficiency of the column

An estimate of percentage recovery for 5-HTP and 5-HIAA was calculated by first injecting an aqueous standard then an actual brain sample which contained 5-HTP or 5-HIAA, and finally a brain sample spiked with aqueous standard (see Table 21).

Table 21.

Efficiency of the column for detecting 5-HTP and 5-HIAA

Peak Height (mm)	5-HTP (n=2)	5-HIAA (n=2)
50% of standard	78±1.5	48±1.0
50% of brain sample	47±0	11±0.5
(50% of brain sample + 50% of standard)	124±2.0	49±2.0
Recovery %	99.2	83

The results are expressed means±s.e.m. of two separate experiments. Reproducibility was very good.

Since the percentage recovery for 5-HTP is approaching 100%, no further correction to its values was necessary, while those of 5-HIAA were adjusted accordingly.

The peaks of interest were susceptible to displacement by varying the mobile phase composition. Untreated brain samples produced little or no peaks at the retention time of 5-HTP. The peaks produced by a spiked brain sample were superimposed on and additive to those of treated brain samples. Furthermore, the injection of an unspiked sample of Milli Q water failed to produce any peaks, confirming that no impurity was present in the water. These findings corroborate and add credence to the idea that the peaks being measured truly belonged to 5-HTP and 5-HIAA.

4.2.9 Estimation of Protein content

In order to obtain an estimation of the protein content of cerebral cortex tissue, the Lowry *et al.* (1951), protein assay was employed, using bovine serum albumin as standard (Table 22).

Table 22.

Estimation of protein content of rat cerebral cortex

Protein Conc.	Absorbance	
20 µg	0.097	{Unknown-1 0.589 = 108.5 µg}
60 µg	0.324	{Unknown-2 0.598 = 110 µg}
100 µg	0.565	{Unknown-3 0.598 = 110 µg}
120 µg	0.658	

Unknown 1-3 contain 0.84 mg cerebral cortex tissue = 109.5 µg protein, or 1 mg of cerebral cortex contains 0.13 mg protein.

The mean weight of cerebral cortex tissue obtained from each rat was 382 mg. This means that the mean protein obtained from each rat was approximately 50 mg.

4.2.10 Calculations and Statistics

The effect of 8-OH-DPAT on 5-HTP concentrations is expressed as percentage formation, with respect to control values. They were calculated assuming a maximum effect at 300 µg/kg of 8-OH-DPAT. Basal 5-HTP values being first deducted from mid-dark control and drug treated values.

Each set of data was first analysed by means of one-way analysis of variance followed by Dunnett's t-test to assess the differences in the means of the treatments as compared to control. Student's t-test was then applied to compare for statistical differences between mid-light and mid-dark at each dose of 8-OH-DPAT.

4.2.11 Drugs

m-hydroxybenzylhydrazine dihydrochloride (Sigma, St. Louis, MO, USA), 8-hydroxy-2-(di-n-propylamino) tetralin (Semat Technical Ltd., St. Albans, England), 5-

hydroxyindole acetic acid (Sigma), 5-hydroxytryptophan (Sigma).

4.3 RESULTS

The sensitivity for detecting 5-HTP was set at a peak/noise ratio of 2 (*i.e.* a peak whose height is double the baseline noise), in the present case this was 35 pg/100 µl sample (*i.e.*3.5 ng/g 5-HTP). Experiments to determine endogenous, basal concentrations of 5-HTP (*i.e.* in untreated rats) revealed that very little was actually present. Indeed, concentrations observed at mid-light were below the level of detection. However, concentrations at mid-dark were slightly higher, approximating to a mean of about 50 pg/100 µl sample (*i.e.* 5.0 ng/g 5-HTP). This finding is rather surprising since 5-HTP is considered to be decarboxylated almost immediately after synthesis. These results suggest that the difference in 5-HTP concentrations may be the consequence of a variation in tryptophan hydroxylase or 5-HTP decarboxylase activity.

Earlier studies carried out in our laboratory (Sinei, 1987) to monitor 5-HTP concentrations, 60 min after 5-HTP decarboxylase inhibition, have shown the rate of formation of 5-HTP to depend on the dose of NSD 1015 used. This rate of formation of 5-HTP was linear up to a dose of 50 mg/kg; higher doses of 75 and 100 mg/kg did not result in any further increases in 5-HTP. A dose of 100 mg/kg was therefore considered suitable to achieve adequate decarboxylase inhibition. Using this dose it was shown that the accumulation of 5-HTP was linear with time for about 60 min, after which it was observed to fall. This fall was attributed to either the recovery of the enzyme or the redistribution of the accumulated 5-HTP away from the brain. The work of Hjorth *et al.* (1982) using similar doses has shown that the optimum time to observe 5-HTP concentrations is 60 min after 8-OH-DPAT or 30 min after NSD 1015, as 5-HTP concentrations begin to recover after that time.

Fig. 39, represents a typical chromatogram obtained with samples from rats given NSD 1015, 30 minutes before death. Each sample took approximately 70 min to elute and for the baseline to return to normal. Although, 5-HTP, 5-HIAA and 5-HT were all detectable and well resolved from each other, only 5-HTP and 5-HIAA were quantified, because the 5-HT peaks were regularly off scale.

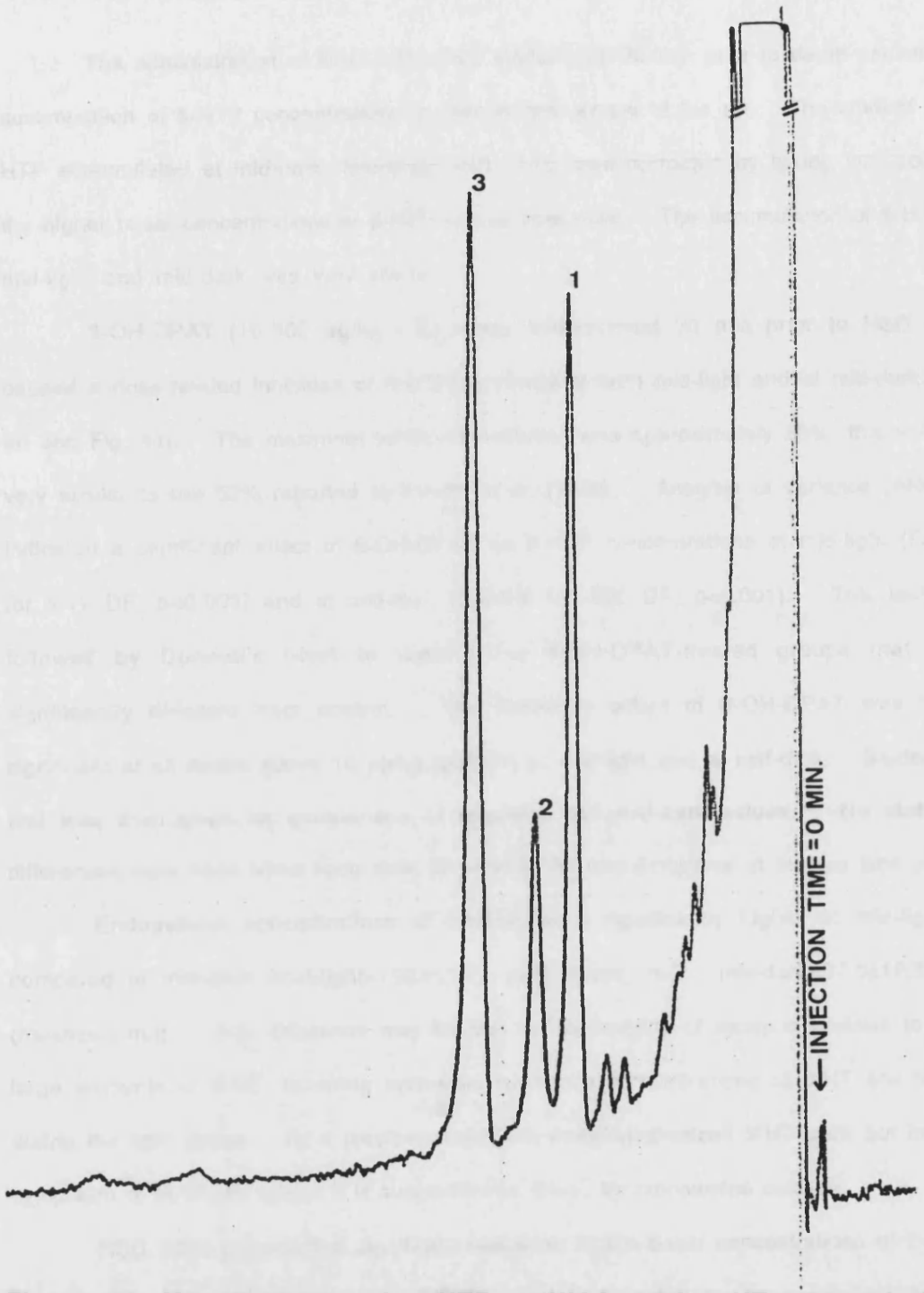


Figure 39. Measurement of 5-HTP, 5-HIAA and 5-HT concentrations using HPLC

This is a typical chromatogram of the rat cortical extracts following a dose of NSD 1015 (100 mg/kg i.p.), 30 minutes before death. The detector potential was set at +0.6 V and sensitivity 0.5 nA f.s.d. The mobile phase and other conditions are described more fully in section 4.2.1. Peak 1 (5-HTP) t_R =19.15 min/sec, Peak 2 (5-HIAA) t_R =21.40 and Peak 3 (5-HT) t_R =27.10.

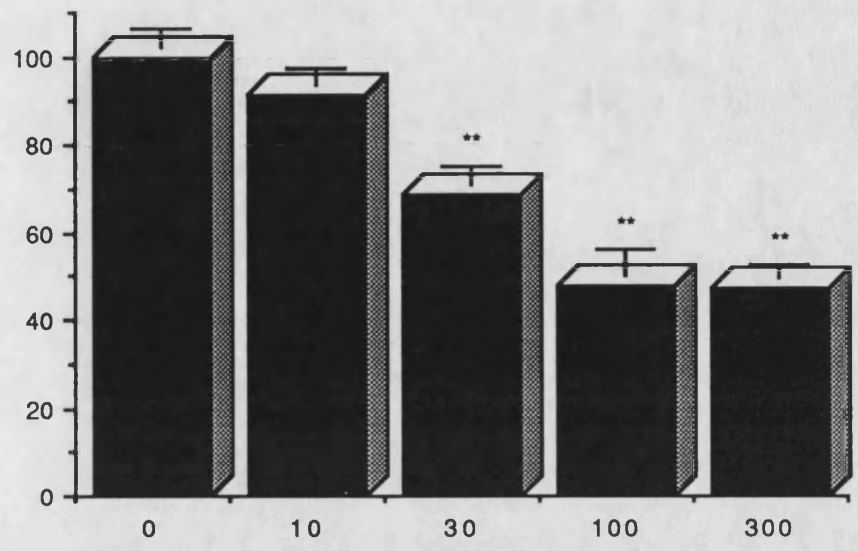
The administration of NSD 1015 (100 mg/kg i.p.), 30 min prior to death caused the accumulation of 5-HTP concentrations in the cerebral cortex of the rat. The amount of 5-HTP accumulated at mid-dark, following NSD 1015, was corrected by taking into account the higher basal concentrations of 5-HTP at this time point. The accumulation of 5-HTP at mid-light and mid-dark was very similar.

8-OH-DPAT (10-300 µg/kg s.c.) when administered 30 min prior to NSD 1015 caused a dose-related inhibition of 5-HTP synthesis at both mid-light and at mid-dark (Fig. 40 and Fig. 41). The maximum inhibition achieved was approximately 53%; this value is very similar to the 52% reported by Hjorth *et al.* (1982). Analysis of variance (ANOVA) indicated a significant effect of 8-OH-DPAT on 5-HTP concentrations at mid-light ($F=64.9$ for 4/17 DF; $p<0.001$) and at mid-dark ($F=30.9$ for 4/25 DF; $p<0.001$). This test was followed by Dunnett's t-test to identify the 8-OH-DPAT-treated groups that were significantly different from control. The inhibitory effect of 8-OH-DPAT was highly significant at all doses above 10 µg/kg ($p<0.01$) at mid-light and at mid-dark. Student's t-test was then used for comparison of mid-light and mid-dark values. No statistical differences were seen when each dose of 8-OH-DPAT was compared at the two time points.

Endogenous concentrations of 5-HIAA were significantly higher at mid-light as compared to mid-dark {mid-light=192.4±19.2 ng/g tissue, n=3 ; mid-dark=97.0±10.3, n=5 (mean±s.e.m.)}. This difference may be due to the inability of synaptic vesicles to store large amounts of 5-HT, following synthesis, as tissue concentrations of 5-HT are highest during the light phase. As a result of which the newly-synthesized 5-HT spills out into the cytoplasm of terminals where it is susceptible to attack by monoamine oxidase.

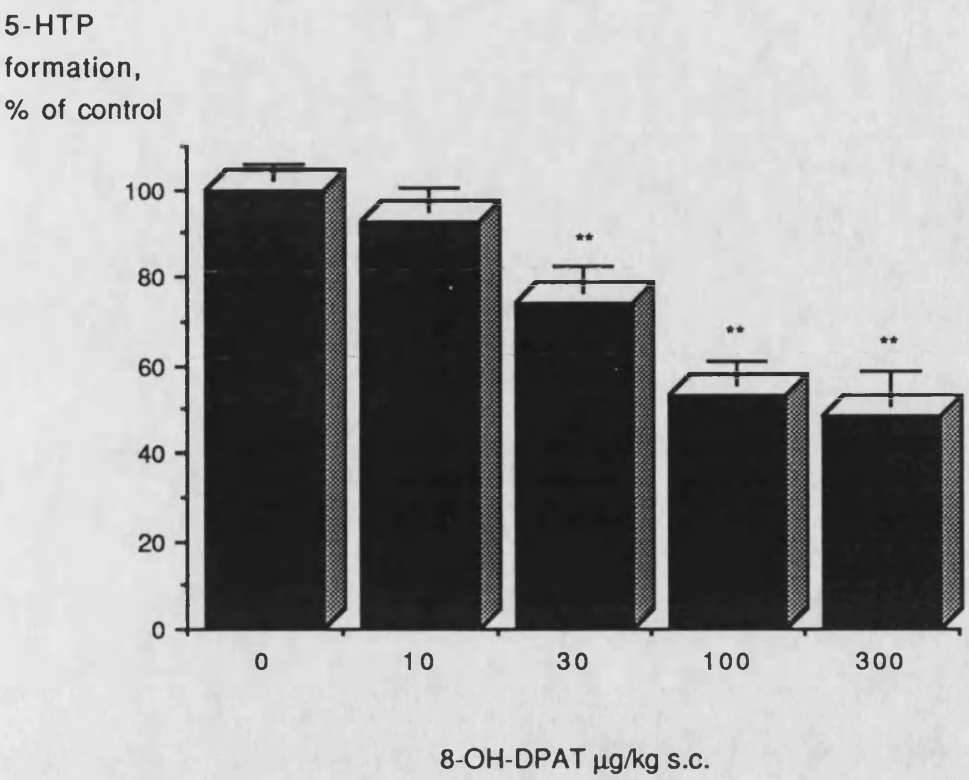
NSD 1015 produced a significant reduction in the basal concentrations of 5-HIAA, 30 minutes after injection, both at mid-light and at mid-dark. Moreover, 8-OH-DPAT produced a similar effect which was additive to that produced by NSD 1015. This effect of 8-OH-DPAT was statistically significant when tested by ANOVA at mid-light ($F=6.8$ for 4/17 DF; $p<0.01$) and at mid-dark ($F=12.5$ for 4/25 DF; $p<0.01$). Further analysis using Dunnett's t-test suggested that while only the highest dose of 8-OH-DPAT produced a significant lowering of 5-HIAA concentrations at mid-light (Fig. 42), whereas all doses above 10 µg/kg caused a significant lowering of 5-HIAA concentrations, at mid-dark

Figure 40.



Effect of 8-OH-DPAT on the formation of **5-HTP** in the cerebral cortex of the rat, at **mid-light**. 8-OH-DPAT (0-300 µg/kg s.c.) and NSD 1015 (100 mg/kg i.p.) were given 60 and 30 min, respectively, before death. The results are expressed as percentage inhibition of control value. The endogenous basal concentration of 5-HTP was below the level of detection (*i.e.* less than 3.5 ng/g 5-HTP). The mean ± s.e.m. (n=4), control value was 104.5 ± 4.1 ng/g 5-HTP. ANOVA was used to compare control and increasing doses of 8-OH-DPAT, followed by Dunnett's t-test ($^{**}p<0.01$).

Figure 41.

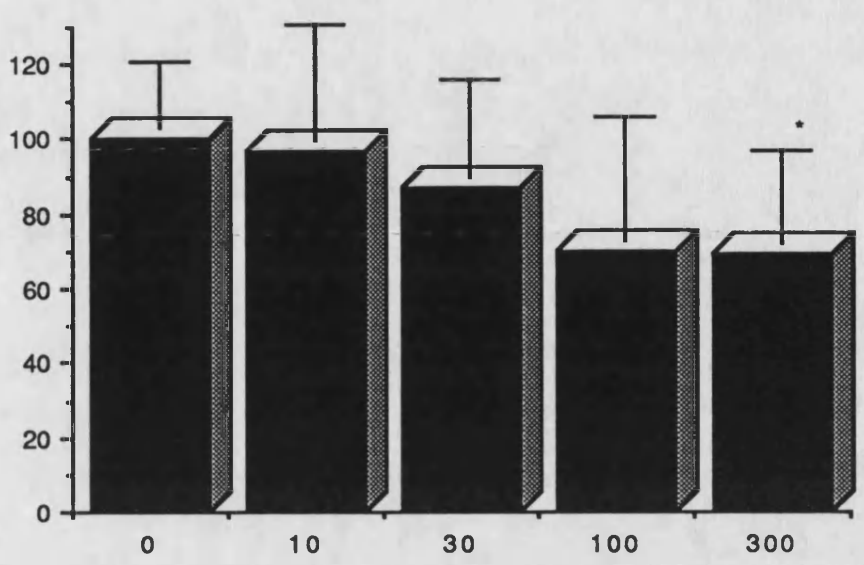


Effect of 8-OH-DPAT on the formation of **5-HTP** in the cerebral cortex of the rat, at **mid-dark**. 8-OH-DPAT (0-300 µg/kg s.c.) and NSD 1015 (100 mg/kg i.p.) were given 60 and 30 min, respectively, before death. The results are expressed as percentage inhibition of control value. The endogenous basal concentration of 5-HTP was 5 ± 0.5 ng/g 5-HTP. The mean ± s.e.m. (n=6), control value was 103.5 ± 3.7 ng/g 5-HTP. ANOVA was used to compare control and increasing doses of 8-OH-DPAT, followed by Dunnett's t-test ($^{**}p<0.01$).

(Fig. 43). No statistical differences were observed when comparisons were made between each doses of 8-OH-DPAT, at the two time points.

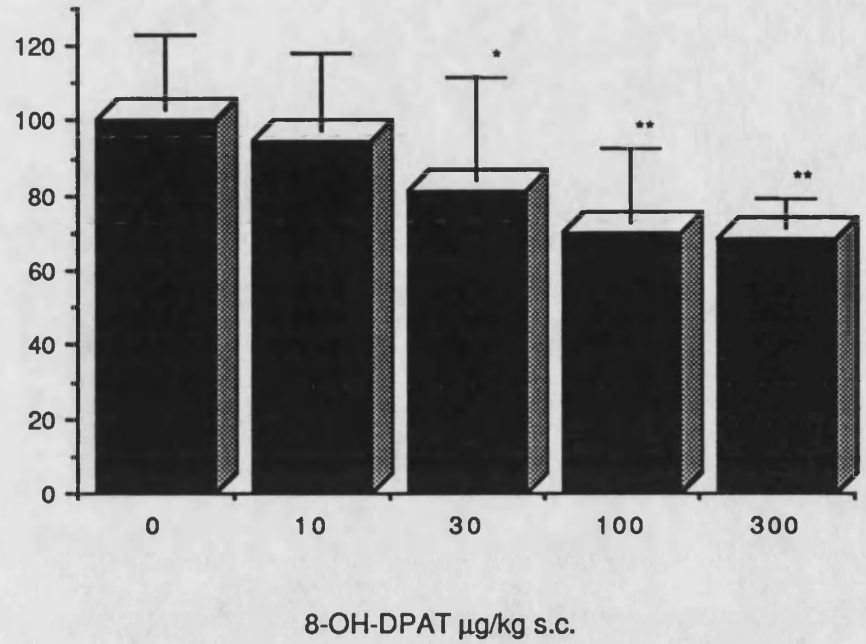
The results from this section show that 8-OH-DPAT reduces the rate of 5-HT synthesis (as measured by the inhibition of 5-HTP formation), to an extent which is the same at mid-light and mid-dark. NSD 1015 and 8-OH-DPAT both caused a significant reduction in 5-HIAA concentrations; this effect is probably secondary to their effect on 5-HT synthesis.

Figure 42.



Effect of 8-OH-DPAT on the formation of **5-HIAA** in the cerebral cortex of the rat, at **mid-light.** 8-OH-DPAT (0-300 µg/kg s.c.) and NSD 1015 (100 mg/kg i.p.) were given 60 and 30 min, respectively, before death. The results are expressed as percentage inhibition of control value. The mean ± s.e.m. (n=4), control value was 49.7 ± 1.8 ng/g 5-HIAA. ANOVA was used to compare control and increasing doses of 8-OH-DPAT, followed by Dunnett's t-test ($^{*}p<0.05$).

Figure 43.



Effect of 8-OH-DPAT on the formation of **5-HIAA** in the cerebral cortex of the rat, at **mid-dark**. 8-OH-DPAT (0-300 µg/kg s.c.) and NSD 1015 (100 mg/kg i.p.) were given 60 and 30 min, respectively, before death. The results are expressed as percentage inhibition of control value. The mean ± s.e.m. (n=6), control value was 51.8 ± 2.1 ng/g 5-HIAA. ANOVA was used to compare control and increasing doses of 8-OH-DPAT, followed by Dunnett's t-test ($^{*}p<0.05$, $^{**}p<0.01$).

4.4 DISCUSSION

Circadian variations are reported in 5-HT concentrations in the brain stem (Semba *et al.*, 1984 ; Agren *et al.*, 1986) and terminal-containing regions (Quay, 1968 ; Hery *et al.*, 1972 ; Semba *et al.*, 1984), with higher concentrations being observed during the light phase of the light : dark cycle. In contrast, extracellular concentrations of 5-HT, when measured by brain microdialysis, are higher during the dark period, suggesting that the release of 5-HT into the synaptic cleft is greater at this time (Martin and Marsden, 1985 ; Kalen *et al.*, 1988,1989). In support, electrophysiological studies have shown that the firing rate of 5-HT neurons in freely moving cats is increased during arousal and decreased during drowsiness and sleep (McGinty and Harper, 1976 ; Trulson and Jacobs, 1979). These studies are consistent with the idea that the greater release of 5-HT during the dark phase, may result from the higher firing rate of 5-HT neurones associated with an increase in the arousal or vigilance of the animals (Kalen *et al.*, 1989).

The firing rate of these neurones is controlled by many factors, including the activity of the somadendritic 5-HT autoreceptor, which when stimulated by 5-HT or 5-HT_{1A}-selective agonists, inhibits 5-HT neuronal firing (Sprouse and Aghajanian, 1986,1987), and, as a consequence, the synthesis (Hjorth *et al.*, 1982) and the release (Garratt *et al.*, 1988) of 5-HT. This somadendritic 5-HT autoreceptor is believed to be of the 5-HT_{1A} subtype (see section 1.4.9), although a pharmacological characterization can not be performed due to the lack of a suitable 5-HT_{1A} antagonist. However, a recent report suggests that a fluoro analogue of 8-OH-DPAT may possess 5-HT_{1A} antagonist activity, and this compound may prove useful in the characterization of this receptor (Hillver *et al.*, 1990).

The ability of the somadendritic 5-HT autoreceptor to control the firing rate of 5-HT neurones suggests that a variation in its sensitivity, or activity, may account for the observed circadian variation in synthesis and release of 5-HT. The aim of this study was to investigate the sensitivity of this autoreceptor at mid-light and at mid-dark, corresponding to time points when the firing rate of 5-HT neurones would be expected to be lowest and highest respectively.

The sensitivity of the somadendritic 5-HT autoreceptor was measured by the

ability of 8-OH-DPAT (which at low doses preferentially activates this receptor) to attenuate 5-HT synthesis (as measured by the accumulation of 5-HTP, after 5-HTP-decarboxylase inhibition) in the cerebral cortex, resulting from its inhibitory effect on 5-HT neuronal firing (Sprouse and Aghajanian, 1986, 1987 ; Blier and De Montigny, 1987).

The endogenous basal concentration of 5-HTP in the cerebral cortex was higher at mid-dark (about 50 pg/100µl) as compared to mid-light, when it was below the level of detection (*i.e.* less than 35 pg/100µl). This variation in 5-HTP concentrations is unlikely to result from a variation in 5-HTP-decarboxylase activity, since this enzyme is normally unsaturated with its substrate (Hillier and Redfern, 1976a). However, these authors observed a variation in 5-HTP-decarboxylase activity when using crude brain homogenates, but were unable to observe any differences after purification of the enzyme, suggesting that the variation in the activity of the enzyme was the result of other factors, such as substrate competition or co-factor availability. The greater 5-HTP concentrations observed during mid-dark may be caused by the higher rate of 5-HT synthesis, reported at this time point (Fernstrom and Wurtman, 1971 ; Sinei, 1987). It is also possible that this phenomena could be caused by the enzyme, 5-HTP-decarboxylase approaching saturation with its substrate or from co-factor limitation.

The administration of NSD 1015 (5-HTP decarboxylase inhibitor) was associated with an accumulation of 5-HTP in the cerebral cortex; the concentrations observed during the light and the dark phase were the same. Increasing doses of 8-OH-DPAT produced a dose-dependent inhibition of the accumulation of 5-HTP. The size of the inhibition was essentially identical at each dose, when its effect at mid-light was compared with that at mid-dark. In addition, 8-OH-DPAT caused a dose-dependent reduction of 5-HIAA concentrations, the magnitude of which was similar at each dose, during mid-light and mid-dark. This reduction in 5-HIAA concentrations is likely to reflect either the decreased metabolism, resulting from a decrease in the release of 5-HT (Hjorth *et al.*, 1982), or the decreased intraneuronal metabolism of 5-HT. These results demonstrate that the sensitivity of the somadendritic 5-HT autoreceptor to 8-OH-DPAT is unchanged when measured at mid-light or at mid-dark. These results also suggest that the diurnal variation in the 5-HT neuronal firing rate is not derived from an innate rhythm in the activity of this receptor, although the autoreceptor sensitivity would have to be

determined at a greater number of time points, during the light : dark cycle, to be absolutely certain. The firing rate of 5-HT neurones is likely to be under the control of other neuronal inputs, such as the locus coeruleus (Baraban and Aghajanian, 1980) or the SCN (Bons *et al.*, 1983).

The firing rate of 5-HT neurones and the release of 5-HT are greater during the dark phase, when the animals are most active (McGinty and Harper, 1976 ; Trulson and Jacobs, 1979 ; Kalen *et al.*, 1989). In addition, 5-HT synthesis, as gauged by the activity of the enzyme tryptophan hydroxylase, is also highest during the dark phase (Redfern and Sinei, 1985). However, the rate of 5-HT synthesis in terminal-containing regions can be modulated by the 5-HT neuronal firing rate as well as by local mechanisms (Hjorth and Magnusson, 1988). Evidence for a role of local mechanisms has been provided by experiments where the rate of 5-HT synthesis, following unilateral axotomy, was not significantly different on the transected and the intact sides (Hjorth and Magnusson, 1988). These authors proposed two equally plausible explanations to account for their results: i) Transection impairs the propagation of nerve impulses in ascending 5-HT neurones, as well as other neurones, some of which may impinge on and interfere with the activity in 5-HT terminals. A disinhibition of these non-serotoninergic influences on terminal 5-HT synthesis and release may lead to an increase in the rate of 5-HT synthesis. ii) The decrease in 5-HT release following axonal transection would result in an attenuation of the (autoreceptor- and/or postsynaptic 5-HT receptor-mediated) negative feedback mechanism of terminal 5-HT synthesis. In turn, this might counterbalance the expected reduction in synthesis, following the inhibition of neuronal firing. In support of this hypothesis, Sharp *et al.* (1988) reported a small amount of 5-HT release to be still evident, following the administration of 8-OH-DPAT, at a dose which would be expected to produce its maximal effect. Furthermore, in the case of dopamine neurones, the reduced activation of the terminal dopamine autoreceptor, caused by axonal transection, enhances the activity of the enzyme tyrosine hydroxylase (an analogous situation might exist for 5-HT neurones) (Walters and Roth, 1976). Moreover, the highest dose of 8-OH-DPAT (300 µg/kg s.c.) caused a reduction in the rate of 5-HTP synthesis on the transected side; this effect may stem from a loss of selectivity and probably reflects its action on the terminal 5-HT autoreceptor (Hjorth and Magnusson, 1988). In support of this explanation, an *in vitro*

study has reported 8-OH-DPAT to be capable of activating the terminal 5-HT autoreceptor (Hamon *et al.*, 1984).

Apart from local mechanisms, the rate of 5-HT synthesis can be modulated by the 5-HT neuronal firing rate, which requires an intact 5-HT neurone (Hjorth and Magnusson, 1988). However, doses of 8-OH-DPAT which totally inhibit 5-HT neuronal firing, as determined by electrophysiological techniques (Neale *et al.*, 1987), produce a maximum inhibition of only about 53% of 5-HTP synthesis, in the cerebral cortex, and a number of other brain regions (Hjorth *et al.*, 1982). The weaker effect of 8-OH-DPAT on 5-HT synthesis, as compared to its potent effect on the firing rate of 5-HT neurones, suggests that about 47% of 5-HT synthesis takes place independently of the firing rate. This firing-insensitive component of 5-HT synthesis is probably controlled by local mechanisms, similar to those responsible for the enhancement of 5-HT synthesis following neuronal transection. The results presented here confirm that a proportion of 5-HT synthesis can take place following the administration of 8-OH-DPAT, at doses which totally inhibit the firing rate of 5-HT neurones. This rate of 5-HT synthesis was the same, during mid-light and mid-dark. It is likely therefore that the firing rate of 5-HT neurones partially modulates the synthesis of 5-HT, and this component may be additive on the synthesis that takes place when the 5-HT neuronal firing is inhibited.

The firing rate of 5-HT neurones is reported to be dependent on the state of arousal or vigilance of the animals (McGinty and Harper, 1976 ; Trulson and Jacobs, 1979), which can in turn influence the rate of 5-HT synthesis (Hjorth and Magnusson, 1988). It is possible therefore that any factor/s that change the firing rate will produce an accompanying change in the rate of 5-HT synthesis. In the present study it was necessary to disturb the animals, from their normal sleep/wake cycle, for the administration of drugs. As a consequence, the animals in their active phase (dark phase) underwent handling and injections, while those in their rest phase (light phase) underwent the additional stress of being woken up, prior to handling and injection. Handling and injection are stressful stimuli which are reported to be associated with an increase in 5-HT release; handling (72% increase), tail pinch (48% increase) (Kalen *et al.*, 1989). This increase in 5-HT release is likely to result from a corresponding increase in the firing rate of their 5-HT neurones. In addition, the awakening of previously asleep animals is associated with an

increase the firing rate of their 5-HT neurones (Trulson and Jacobs, 1979). It is likely that these unavoidable interferences caused a change of the rate of synthesis of 5-HT, in terminal containing regions. A higher level of change, in 5-HT synthesis, would be expected in animals that were at the mid-light point of their light : dark cycle, because the firing rate of their 5-HT neurones is generally low at this time (McGinty and Harper, 1976). Furthermore, it is possible that the biochemical changes resulting from these interferences may have clouded the observation of a variation in somadendritic 5-HT autoreceptor sensitivity, which may exist under normal physiological conditions.

The somadendritic 5-HT autoreceptor is reported to be capable of undergoing desensitization following drug administration. This desensitization is not apparent in *in vitro* or *in vivo* electrophysiological studies, following acute administration of a 5-HT_{1A} agonist, CM 57493 (Adrien *et al.*, 1989), but can be produced by the long-term administration of gepirone (Blier and De Montigny, 1987). The firing rate of 5-HT neurones was depressed during the acute phase of the long-term administration of the 5-HT uptake inhibitors, indalpine or zimeldine (De Montigny *et al.*, 1984), and also after gepirone (Blier and De Montigny, 1987), but recovered to normal levels after 14 days of their administration. This recovery of the firing rate, to normal levels, is likely to be caused by the desensitization of the somadendritic 5-HT autoreceptor (De Montigny *et al.*, 1984 ; Blier and De Montigny, 1987). However, the long term administration of 8-OH-DPAT was reported not to impair the ability of the somadendritic 5-HT autoreceptor to attenuate the synthesis of 5-HTP, in the rat hippocampus or hypothalamus (Larsson *et al.*, 1990); in support of this, the K_D and B_{max} values of the 5-HT_{1A} binding site in the medulla were unchanged. These findings are rather surprising, since this treatment would have been expected to produce a desensitization of the somadendritic 5-HT autoreceptor, but they add further credence to the idea that the measurement of 5-HTP synthesis, following 5-HTP decarboxylase inhibition, gives a good indication of somadendritic 5-HT autoreceptor sensitivity.

The hyperphagic behavioural response in satiated rats is attributed to the selective activation of the somadendritic 5-HT autoreceptor, and exhibits rapid desensitization following the single, low dose injection of 8-OH-DPAT (Kennett *et al.*, 1987). In addition, this hyperphagic response of 8-OH-DPAT is prevented by pretreatment with PCPA (Hutson

et al., 1987), a treatment reported to cause desensitization of the somadendritic 5-HT autoreceptor (Chaput *et al.*, 1987). However, a rapid desensitization of the somadendritic 5-HT autoreceptor is not observed in electrophysiological studies (Adrien *et al.*, 1989). These authors proposed that the reduction in the hyperphagic response may depend on other mechanisms, rather than an adaptive change of the somadendritic 5-HT autoreceptor or that the autoreceptor involved in the behavioural effects of 5-HT_{1A} agonists is different from that responsible for the inhibition of 5-HT neuronal firing. Furthermore, Sleight *et al.* (1988) demonstrated that 8-OH-DPAT was capable of producing both hyperphagia and hypophagia in freely feeding rats, depending on its time of administration. They attributed this mixed effect of 8-OH-DPAT to be caused by the existence of two distinct 5-HT systems modulating food intake, one mediating hyperphagia (central) and the other mediating hypophagia (peripheral).

In conclusion, it is clear that the somadendritic 5-HT autoreceptor, by its ability to modulate the firing rate of 5-HT neurones, regulates the synthesis and the release of 5-HT in terminal containing regions. The firing rate of these neurones and the release of 5-HT is greater during the dark phase, when the rats are more active. In addition, the rate of 5-HT synthesis is also at its highest during the dark phase. It is tempting to speculate that the greater 5-HT synthesis during the dark phase is caused by the higher firing rate at this time. The rate of 5-HT synthesis can be modulated partially by the firing rate and partially by local mechanisms (*i.e.* independent of 5-HT neuronal firing). The results presented in this study demonstrate that the sensitivity of the somadendritic 5-HT autoreceptor to 8-OH-DPAT, as measured by the ability of this compound to depress 5-HT synthesis, was the same at mid-dark and at mid-light. These findings suggest that factors other than any variation in the sensitivity of the somadendritic 5-HT autoreceptor are responsible for the observed circadian variation in the synthesis of 5-HT, although the sensitivity of the somadendritic 5-HT autoreceptor would have to be determined at several more time points before a definite conclusion could be drawn about any circadian changes in its sensitivity. Furthermore, these results imply that the changes observed in the firing rate of 5-HT neurones, during the light : dark cycle, are not caused by an altered sensitivity of the somadendritic 5-HT autoreceptor, however, it would be capable of passing on any fluctuations applied to it. Clearly, more studies are required to determine

the underlying factors responsible for the firing rate of 5-HT neurones, and also for the identification of components that may exercise control over it.

5 GENERAL DISCUSSION

6 GENERAL DISCUSSION

The results presented in this thesis have already been extensively discussed in each of the relevant chapters. The purpose of this section is to provide general comment on the techniques used, and to suggest areas where further investigation would be particularly beneficial.

Biochemical techniques were employed in this thesis to determine the functional activity of 5-HT autoreceptors modulating the release and synthesis of 5-HT during the 12h light : 12h dark cycle.

From the results described in this thesis it can be concluded that the circadian variation in the release of 5-HT from central serotoninergic neurones, is not the result of a corresponding rhythm in the sensitivity of the terminal 5-HT_{1B} autoreceptor (rat), terminal 5-HT_{1D} autoreceptor (guinea-pig) or the presynaptic *alpha*$_2$-adrenoceptor located on serotoninergic nerve terminals. In addition, the circadian rhythm in 5-HT synthesis is not derived from a corresponding rhythm in the sensitivity of the somadendritic 5-HT_{1A} autoreceptor.

The superfusion technique employed in this thesis for the estimation of the sensitivity of receptors located on 5-HT terminals in regulating the release of 5-HT has a number of drawbacks. It primarily relies on the release of previously taken up [^{3}H]5-HT as an indicator of the release of endogenous 5-HT, but it is not known how evenly this ligand mixes with the endogenous stores of 5-HT or if the release consists of [^{3}H]5-HT. Furthermore, the method of stimulation used is rather unspecific in that the whole slice is stimulated, and not just the neurones containing 5-HT. This mode of stimulation is likely to cause the release not just of 5-HT but also of a host of other neurotransmitters, some of which may, by their receptors, or through other mechanism/s, be capable of regulating the release of 5-HT. It is likely therefore that the overall release of tritium evoked by this method of stimulation, and any drug-induced changes in the release rate, are the result of complex interactions between neurotransmitters. In addition, the use of an artificially high level of stimulation to evoke neurotransmitter release, when used in conjunction with a 5-HT uptake inhibitor, leads to an elevation in synaptic 5-HT concentrations. This

condition is likely to cause a much greater (and prolonged) stimulation of the terminal 5-HT autoreceptor than would be expected under normal physiological conditions, and, probably accounts for the discrepancy between functional potencies obtained using this technique when compared with those of radio-ligand binding studies. Under such circumstances, it is not certain that all the systems usually operating to control the release of neurotransmitter are functioning normally. It is also possible that circadian variations that are important physiologically become obscured. For example, the *in vivo* release of 5-HT is greater during the dark phase (Martin and Marsden, 1985 ; Kalen *et al.*, 1989) but this variation was absent in the present *in vitro* studies. There is no way to control for all of these variables except by studying the 5-HT autoreceptor by a number of methods, each of which will have their own particular drawbacks.

The study of neurotransmitter release from synaptosomes is useful in that it allows the estimation of the sensitivity of the 5-HT autoreceptor in the absence of an endogenous inhibitory tone, but at the expense of the loss of structural integrity of the tissue. A more physiological technique, recently described (Blier *et al.*, 1989b), uses a low frequency of stimulation to minimize feedback inhibition (see section 2.5), but it still relies on the measurement of the release of [^{3}H]5-HT as an indicator of 5-HT release. A further advancement would be made if this technique could be applied to the measurement of the release of endogenous 5-HT.

In vitro superfusion techniques provide only a "snap shot" estimation of the receptor activity at one particular time point. It is possible that a rhythm could be missed if it was of a low amplitude or if the sampling times did not correspond to the zenith and nadir of its activity. A way of discounting this possibility would be to increase the number of sampling times during the light : dark cycle. However, this would still not be as good as a continuous *in vivo* technique which could provide information of the on-going changes in the activity of the terminal autoreceptor during the light : dark cycle. The development of such a technique is eagerly awaited.

The results of functional studies could not be correlated with those of radio-ligand binding studies because of the poor choice of displacing agent and also because the 5-HT$_{1B}$ binding sites are located pre- and post-synaptically (Crino *et al.*, 1990). The

presynaptically located 5-HT_{1B} binding sites are considered to correspond to the terminal 5-HT autoreceptor in the rat brain (Verge *et al.*, 1986). These binding sites are likely to be present in small numbers, and this has hindered their quantification (Verge *et al.*, 1985 ; Weissmann-Nanopoulos, 1985). In addition, an inherent limitation of radio-ligand binding studies is their inability to distinguish between functional and non-functional receptors. Furthermore, this technique would be incapable of detecting the decoupling of a receptor from its secondary messenger system. One possible approach to this problem would be to study the 5-HT receptor-stimulated secondary messenger system. However, this may pose a problem in brain regions which contain a mixed population of 5-HT receptor subtypes linked to the same secondary messenger system. The development of compounds with increased selectivity for the 5-HT_{1B} and the 5-HT_{1D} autoreceptor will prove to be very useful in this respect. Experiments of this type will undoubtedly complement the results of this thesis, and shed further light on the mechanisms involved in the regulation of 5-HT release.

The rate of 5-HT synthesis in terminal containing regions is controlled partially by the firing rate of 5-HT neurones, and partially by local mechanism/s which become more prominent when the neuronal firing is impeded (*i.e.* following nerve transection) (Hjorth and Magnusson, 1988). The nature of these local mechanism/s controlling 5-HT synthesis is at present not known but it is thought that the terminal 5-HT autoreceptor may be involved, as is the case for the dopamine autoreceptor (Walters and Roth, 1976). Further studies in this area will serve to identify the factors that are able to control the rate of 5-HT synthesis, and of the mechanism/s involved. These studies, and many more, will serve to increase our understanding of the factors that are ultimately responsible for the circadian variation in 5-HT concentrations.

In the present study the sensitivity of the somadendritic 5-HT autoreceptor was assessed by its ability to modulate 5-HT synthesis, as a consequence of its effect on neuronal firing. A far superior, and probably more accurate method of estimating the *in vivo* sensitivity of this receptor would be to monitor directly the effect of the microiontophoretic application of compounds on the firing rate of 5-HT neurones, during the light : dark cycle. This method would also bypass any fluctuations which may be caused by

diurnal differences in the pharmacokinetic handling of the compounds.

It would also be of interest to determine the factors that are responsible for initiating and controlling the firing rate of 5-HT neurones, since the firing rate is likely to be the major determinant of the release of 5-HT in terminal containing regions. It would be interesting to correlate the *in vivo* firing rate of 5-HT neurones with the simultaneous measurement of the release of 5-HT by microdialysis in terminal containing regions, during the light : dark cycle. However, an inherent problem with microdialysis is its poor recovery of neurotransmitters; as a consequence sampling times are long, and this would be expected to even out any circadian variations. A way around this problem would be to monitor extracellular 5-HT concentrations using *in vivo* voltammetry (Crespi *et al.*, 1988). This technique will provide for shorter sampling times and continuous recording, thus enabling better correlations of neurotransmitter release with on-going behaviour.

As reported here, many of the components involved in the synthesis of 5-HT display a variation in their function, during the light : dark cycle. In addition, the behavioural effects of 5-HT receptor stimulation also display a circadian variation. From the available evidence it is possible to propound a hypothesis to explain how the various components of 5-HT neuronal firing, release and synthesis interact to produce the circadian variation in 5-HT concentrations.

The circadian variation in the release of 5-HT can be explained by the corresponding variation in the firing rate of 5-HT neurones, both are greater during the dark phase. However, a similar relationship between the firing rate and the circadian variation in 5-HT concentrations in terminal containing regions is not so readily apparent. The firing rate of 5-HT neurones can modulate the rate of 5-HT synthesis. In addition, 5-HT synthesis can occur independently of the firing rate of 5-HT neurones, and is probably controlled by local mechanisms. It is tempting to speculate that the higher firing rate of 5-HT neurones, during the dark phase, may be responsible for stimulating the "firing-dependent" component of 5-HT synthesis, while the lower firing rates observed during the light phase would produce a comparatively smaller rise in the rate of 5-HT synthesis. In contrast, the "firing-insensitive" component of 5-HT synthesis would proceed at a steady pace during the light : dark cycle. These two components of 5-HT synthesis are probably

additive, therefore it is possible that any changes observed in the 5-HT synthesis rate are the direct result of a variation in the "firing-sensitive" component. In support of a greater rate of 5-HT synthesis during the dark phase is the higher activity of tryptophan hydroxylase, higher brain concentrations of its precursor, tryptophan, and the observation that newly synthesized 5-HT is released preferentially to old. On this basis, the higher firing rate of 5-HT neurones during the dark phase accounts for the the greater release and synthesis of 5-HT. However, these parameters are 180° out of phase with 5-HT concentrations. It is possible that the lower firing rate and consequently the lower release rate observed during the light phase, enable the 5-HT synthesized by this "firing-insensitive" component to accumulate and thus account for the higher concentrations observed during the light phase.

This theory, like all theories, is based on the current knowledge in this area. It is hoped that this theory will stimulate further research, which will serve to modify and correct it.

In conclusion, the results presented in this thesis demonstrate that the circadian variation in the release and the synthesis of 5-HT is not due to an innate rhythm in the sensitivity of the terminal- or the somadendritic-5-HT autoreceptor respectively. In addition, there are as yet no suitable behavioural models for assessing the activity of the 5-HT autoreceptor, but the development of such models in the future is likely to prove useful for complementing the *in vitro*/*in vivo* results presented in this thesis.

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